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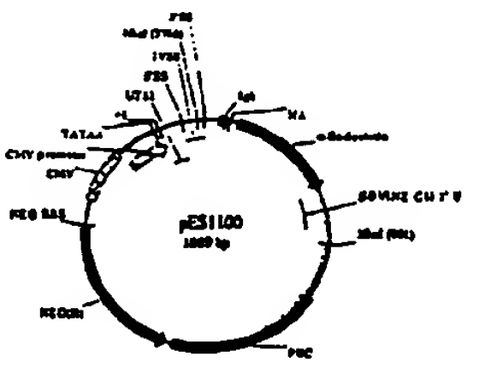
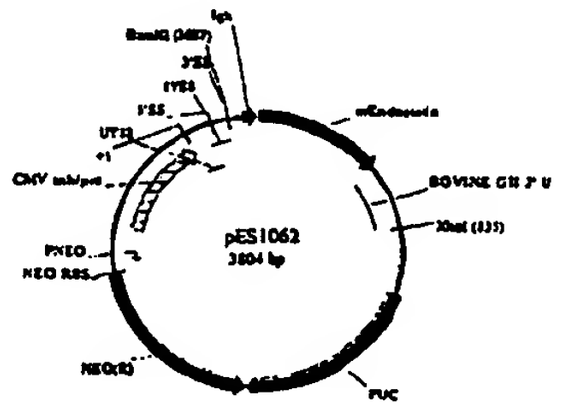
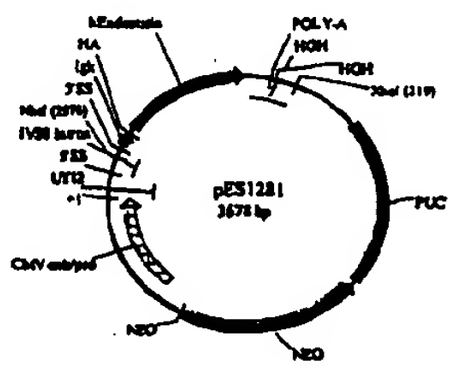
(57) Abstract

The present invention relates to gene delivery and gene therapy, and provides novel nucleic acid constructs for expression of anti–angiogenic agents in a mammal, formualtions for delivery that incorporate a nucleic acid construct for expression, and methods for preparing and using such constructs and formulations. In particular, this invention relates to plasmid constructs for delivery of therapeutic anti–angiogenic encoding nucleic acids to cells in order to modulate tumor activity, methods of using those constructs (including combination therapy with other agents, such as cytokines, preferably IL–12), as well as methods for preparing such constructs.







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DESCRIPTION

Anti-Angiogenesis Plasmids And Delivery Systems, And Methods Of Making And Using The Same

Field Of The Invention

The present invention relates to gene delivery and gene therapy, and provides novel nucleic acid constructs for expression of anti-angiogenic gene products in a mammal, formulations for delivery that incorporate a nucleic acid construct for expression, and methods for preparing and using such constructs and formulations.

Background Of The Invention

The following discussion of the background of the invention is merely provided to aid the reader in understanding the invention and is not admitted to describe or constitute prior art to the present invention.

In tumor-bearing hosts, the lack of an effective immune response is due to weak tumor antigenecity or a tumor-immunosupressive environment. Immunotherapy is to strengthen the tumor-host interaction by introduction of tumor-specific antigens or to boost immune function by local cytokine expression. Several cytokine gene medicines such as IL-2, IFN-α and IL-12 have been developed. By direct intratumoral injection of nucleic acid encoding for IFN-α, IL-2 or IL-12 formulated in a polymeric delivery system, tumor-bearing mice develop an immune response, which leads to inhibition of tumor growth in murine syngeneic tumor model. An alternative approach, anti-angiogenic gene medicine, has been recognized as therapeutic strategy for both immunogenic and non-immunogenic tumors.

Tumors get oxygen and nutrients through the blood vessels. The formation of blood vessels, or angiogenesis, is required for tumor growth and metastases. Moreover,

microvessel density in the tumor show a positive relationship with tumor growth, the risk of metastases, tumor recurrence or death (Folkman, J., *J. Natl. Cancer Inst.*, 82:4-6 (1990); Folkman, *J. Nat. Med.*, 1:27-31 (1995)). The onset of tumor angiogensis could be triggered by an upregulation of angiogenic factors such as VEGF and bFGF or by a downregulation of anti-angiogenic factors such as endostatin, angiostatin and thromospondin-1 (Hanahan, D. & Folkman, J., *Cell*, 86:353-64 (1996)). Thus, reconstitution of angiogenic inhibitors would provide a plausible strategy for cancer therapy (Hori, A., *Cancer Res.*, 51:6180-4 (1991); Kim, K.J., *Nature*, 362:841-4 (1993); O'Reilly, M.S. et al., *Cell*, 79:315-28 (1994); O'Reilly, M. S. et al., *Cell*, 88:277-85 (1997); Clapp, C. et al., *Endocrinology*, 133:1292-9 (1993); Gupta, S.K. et al., *Proc. Natl. Acad. USA*, 92:7799-7803 (1995)).

Angiostatin and Endostatin are two members of an expanding family of proteins that are angiogenesis inhibitors. Angiostatin is an internal proteolytic fragment of mature plasminogen. It contains 4 triple loop disulfide-linked structures, known as kringle domains (Residues 98-440). It has been shown that a form of 3 kringle domain (residues 98-333) is more potent *in vitro* (Cao et al., *The Journal of Biological Chemistry*, 271:29461-29467 (1996)) and *in vivo* (Griscelli et al., *Proc. Natl. Acad. Sci. USA*, 95:6367-6372 (1998)). Endostatin is the C-terminal proteolytic fragment of collagen 18a. Its structure resembles that of E-selectin, an adhesion molecule on endothelial cell (EC) surface (Hohenester, E. et al., *EMBO J.*, 17:1656-64 (1998)).

Both angiostatin and endostatin can inhibit endothelial cell (EC) proliferation *in vitro* and angiogenesis *in vivo*. Moreover, recombinant proteins have been shown to inhibit tumor growth and metastases in mouse models when injected at high doses. Recently, it has been shown that combination of angiostatin and endostatin has synergistic effect in inhibition of tumor growth and metastases (Bachelot, T. et al., *Abstract from AARC*, vol 39, March 1998). It has also

shown that anti-angiogenic therapy can synergize radiation therapy in a murine tumor model (Mauceri, et al., *Nature*, 394:287 (1998)).

Anti-angiogenic therapy targets to EC, but not tumor cells. EC is more accessible to systemically deliveried drugs, thus anti-angiogenic therapy is particularly useful in treatment of disseminated cancer. Moreover, EC is not transformed and anti-angiogenic therapy of an experimental model of cancer does not induce acquired drug resistance. Because angiostatic therapy will require a prolonged maintenance of therapeutic levels *in vivo*, the continuous delivery of a recombinant protein will be expensive and cumbersome.

Summary of the Invention

The present invention relates to gene delivery and gene therapy, and provides novel nucleic acid constructs for expression of anti-angiogenic coding sequences in an organism, preferably a mammal, formulations for delivery that incorporate a nucleic acid construct for expression, and methods for preparing and using such constructs and formulations. In particular, this invention relates to plasmid constructs for delivery of therapeutic genes to cells in order to modulate tumor activity. The invention also provides methods of using those constructs (including combination therapy with other treatment methods, such as radiation therapy, or agents, such as cytokines, preferably IL-12), as well as methods for preparing such constructs. The pharmaceutically acceptable, cost effective and highly efficient delivery system presented herein represents an unanticipated improvement over the art.

A gene therapy approach utilizing an interactive polymeric gene delivery system that increases protein expression by protecting plasmid DNA (pDNA) from nucleases and controlling the dispersion and retention of pDNA in injected tissues has been employed. These polymeric interactive non-

condensing (PINC) systems routinely result in a greater amount of gene expression from tissues as compared to delivery of unformulated plasmid in saline. A plasmid expression system encoding murine IFNα4 or IL-12 formulated as a complex with PVP could induce an anti-tumor immune response following direct injection into subcutaneous murine tumors. By using a plasmid that encodes human insulin-like growth factor-I (hIGF-I) and formulated as a PINC complex, a long duration of production of biologically active human IGF-I has shown *in vivo* following intra-muscular injection. Since intramuscularly delivered gene can achieve a high and persistent expression of therapeutic product in circulating system, it is conceivable that i.m. delivered cancer gene medicines could be used in treatment of disseminated disease, a major limitation of gene therapy.

Alternatively, the administration of a therapeutic gene into selected organs bearing tumor metastases (e.g. lung), would be more suitable for a poorly met clinical need. Gene delivery to the lung for the treatment of genetic defects has been explored in experimental models as well as in clinical trials. By using *N*-[1-(2-3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA)/cholesterol/ plasmid 3:1 positively charged complexes, enhanced pulmonary expression of transgenes was shown following intra-tracheal administration or intravenous injection.

By tail-vein injection of lipid/DNA, detectable levels of human growth hormone (hGH) in serum, human factor IX (hFIX) in plasma and chloramphenicol acetyltransferase (CAT) in the lung and liver were observed with positively charged lipid/plasmid complexes prepared from 400 nm extruded liposomes with a cationic lipid to co-lipid ratio of 4:1 mol/mol. Although the administration of positively charged plasmid/cationic lipid complexes to the lung airways induces a cytokine pattern resembling a Th1 cell phenotype, even in the absence of transgene expression, IL-12 transgene expression in murine lungs following administration of IL-12 plasmid/lipid

complexes inhibits the growth of pulmonary metastases of Renca tumors in syngeneic BALB/c mice.

Use of tumor EC-specific promoters to express anti-angiogenic factors is described herein. At least two strategies were used to select proliferating EC-specific promoters. One is to clone promoters of genes that specifically expressed in tumor EC such as flk-1 and avb3 integrins. Another is to generate chimeric promoters of EC-specific enhancers such as endothelin-1 enhancer and cell-cycle-specific promoters such as cyclin A.

The present invention, by using prototype anti-angiogenic genes, endostatin and angiostatin, demonstrated that anti-angiogenic gene medicine inhibited growth of solid tumor by either intratumoral or intramuscular injection. Anti-angiogenic gene medicines also inhibit lung metastatic tumors after intramuscular or intravenous delivery of formulated angiostatic genes.

Thus, in a first aspect, the invention features a plasmid that contains a tissue specific element and an anti-angiogenic coding sequence. Preferably the tissue specific element is specific for endothelial cells and is transcriptionally linked to the anti-angiogenic coding sequence. As explained in detail below, the plasmid may optionally include transcriptional control sequences such as one or more cytomegalovirus promoter sequences.

The "tissue specific element" may include a promoter, preferably selected from the group consisting of ET-1, flk-1, Alpha-V, Beta-3, ICAM-2, cyc A, E2F1, and cdc6, or may include an enhancer, preferably selected from the group consisting of CMV, four copies of ET-1, and seven copies of ET-1. Examples of EC tissue specific promoters are described in detail below.

The tissue specific element preferably is associated or linked with the anti-angiogenic coding sequence in such a manner that the anti-angiogenic coding sequence expression is enhanced, preferably to a predominant (over 50%) nearly exclusive (about 90% or more) or exclusive level (about 100%),

in the tissue or cells of interest. For example, expression may be enhanced by about two-fold.

As used herein, the term "plasmid" refers to a construct made up of genetic material (i.e., nucleic acids). It includes genetic elements arranged such that an inserted coding sequence can be transcribed in eukaryotic cells. Also, while the plasmid may include a sequence from a viral nucleic acid, such viral sequence does not cause the incorporation of the plasmid into a viral particle, and the plasmid is therefore a non-viral vector. Preferably a plasmid is a closed circular DNA molecule.

"Cytomegalovirus promoter" refers to one or more sequences from a cytomegalovirus which are functional in eukaryotic cells as a transcriptional promoter and an upstream enhancer sequence. The enhancer sequence allows transcription to occur at a higher frequency from the associated promoter.

In this context, "transcriptionally linked" means that in a system suitable for transcription, transcription will initiate under the direction of the control sequence(s) and proceed through sequences which are transcriptionally linked with that control sequence(s). Preferably no mutation is created in the resulting transcript, which would alter the resulting translation product.

The term "coding region" or "coding sequence" refers to a nucleic acid sequence which encodes a particular gene product for which expression is desired, according to the normal base pairing and codon usage relationships. Thus, the coding sequence must be placed in such relationship to transcriptional control sequences (possibly including control elements and translational initiation and termination codons) that a proper length transcript will be produced and will result in translation in the appropriate reading frame to produce a functional desired product.

In a preferred embodiment the "anti-angiogenic coding sequence" encodes a product selected from the group consisting of endostatin, angiostatin, thrombspondin-1, p53, IL-12, IFN-

alpha, truncated tissue factor, an integrin αvβ3 blocking agent, a VHL gene product, a cell cycle-dependant kinase inhibitor, a VEGFr, bFGFr, and a bFGF binding protein and preferably is a synthetic sequence having optimal codon usage (for the organism receiving the plasmid, preferably a human), or semi-optimal codon usage (for the organism receiving the plasmid, preferably a human), or has the nucleotide sequence of any of the plasmids described herein, more preferably plasmid pES1281, pIP1316, pAS1095 or pAS 1096.

An anti-angiogenic coding sequence encodes a product that reduces angiogenesis in the organism of interest, preferably to a significant extent, (for example to an extent that it creates a therapeutic effect) or reduces angiogenesis in an *in vitro* assay.

A particular example of coding regions suitable for use in the plasmids of this invention are the natural sequences coding for anti-angiogenic agents. Thus, in a preferred embodiment coding region has a nucleotide sequence which is the same as the natural nucleotide sequence encoding the anti-angiogenic agent. However, it may be preferable to have an anti-angiogenic coding sequence which is a synthetic coding sequence. In a preferred embodiment, the anti-angiogenic coding sequence is a synthetic sequence utilizing optimal or semi-optimal codon usage.

Thus, a "sequence coding for a human anti-angiogenic agent" or "a human anti-angiogenic coding sequence" is a nucleic acid sequence which encodes the amino acid sequence of a human anti-angiogenic agent, based on the normal base pairing and translational codon usage relationships. It is preferable that the coding sequence encode the exact, full amino acid sequence of the natural human anti-angiogenic agent, but this is not essential. The encoded polypeptide may differ from the natural human anti-angiogenic agent so long as the polypeptide retains anti-angiogenic activity, preferably the polypeptide is at least 50%, 75%, 90%, or 97% as active as natural human anti-angiogenic agent, and more preferably fully

as active as the natural human anti-angiogenic agent. Thus, the polypeptide encoded by the anti-angiogenic coding sequence may differ from a natural human anti-angiogenic agent by a small amount, preferably less than a 15%, 10%, 5%, or 1% change. Such a change may be of one of more different types, such as deletion, addition, or substitution of one or more amino acids.

The term "transcriptional control sequence" refers to sequences which control the rate of transcription of a transcriptionally linked coding region. Thus, the term can include elements such as promoters, operators, and enhancers. For a particular transcription unit, the transcriptional control sequences will include at least a promoter sequence.

The plasmid, in preferred embodiments, may also contain a growth hormone 3′ untranslated region, preferably from a human growth hormone gene.

A "growth hormone 3' untranslated region" is a sequence located downstream (*i.e.*, 3') of the region encoding material polypeptide and including at least part of the sequence of the natural 3' UTR/poly(a) signal from a growth hormone gene, preferably the human growth hormone gene. This region is generally transcribed but not translated. For expression in eukaryotic cells it is generally preferable to include sequence which signals the addition of a poly-A tail. As with other synthetic genetic elements a synthetic 3' UTR/poly(A) signal has a sequence which differs from naturally-occurring UTR elements. The sequence may be modified, for example by the deletion of ALU repeat sequences. Deletion of such ALU repeat sequences acts to reduce the possibility of homologous recombination between the expression cassette and genomic material in a transfected cell.

The plasmid preferably includes a promoter, a TATA box, a Cap site and a first intron and intron/exon boundary in appropriate relationship for expression of the coding sequence. The plasmid may also include a 5' mRNA leader sequence inserted between the promoter and the coding sequence

and/or an intron/5' UTR from a chicken skeletal α-actin gene. Also, the plasmid may have a nucleotide sequence which is the same as the nucleotide sequence of plasmid any of the plasmids described herein.

The plasmid may also include: (a) a first transcription unit containing a first transcriptional control sequence transcriptionally linked with a first 5'-untranslated region, a first intron, a first coding sequence, and a first 3'-untranslated region/poly(A) signal, wherein the first intron is between the control sequence and the first coding sequence; and (b) a second transcription unit containing a second transcriptional control sequence transcriptionally linked with a second 5'-untranslated region, a second intron, a second coding sequence, and a second 3'-untranslated region/poly(A) signal, wherein the second intron is between the control sequence and the second coding sequence; wherein the first and second coding sequences contain a sequence coding for any two different anti-angiogenic agents, preferably angiostatin and endostatin, although other combinations such as IP-10 and endostatin or IP-10 and TSPf are also possible.

The present invention also provides plasmids and related products and methods with a tissue specific element and an anti-angiogenic coding sequence, wherein the anti-angiogenic coding sequence encodes a fusion or hybrid peptide or protein. The fusion or hybrid agent, for example, may be a fusion product of angiostatin and endostatin. Each portion of the fusion product is preferably anti-angiogenic on its own, and has elevated anti-angiogenic effects when presented as part of a fusion or hybrid product.

The term "transcription unit" or "expression cassette" refers to a nucleotide sequence which contains at least one coding sequence along with sequence elements which direct the initiation and termination of transcription. A transcription unit may however include additional sequences, which may include sequences involved in post-transcriptional or post-translational processes. In preferred embodiments, the first

transcriptional control sequence or the second transcriptional control sequence contain one or more cytomegalovirus promoter sequences. The first and second transcriptional control sequences can be the same or different.

A "5' untranslated region" or "5' UTR" refers to a sequence located 3' to promoter region and 5' of the downstream coding region. Thus, such a sequence, while transcribed, is upstream of the translation initiation codon and therefore is not translated into a portion of the polypeptide product.

For the plasmids described herein, one or more of a promoter, 5' untranslated region (5' UTR), the 3' UTR/poly(A) signal, and introns may be a synthetic sequence. In this context the term "synthetic" means that the sequence is not provided directly by the sequence of a naturally occurring genetic element of that type but rather is an artificially created sequence (*i.e.*, created by a person by molecular biological methods). While one or more portions of such a synthetic sequence may be the same as portions of naturally occurring sequences, the full sequence over the specified genetic element is different from a naturally occurring genetic element of that type. The use of such synthetic genetic elements allows the functional characteristics of that element to be appropriately designed for the desired function.

Thus, a "synthetic intron" refers to a sequence which is not a naturally occurring intron sequence but which will be removed from an RNA transcript during normal post transcriptional processing. Such introns can be designed to have a variety of different characteristics, in particular such introns can be designed to have a desired strength of splice site.

In a preferred embodiment the first and second coding regions are coding regions for angiostatin then endostatin in the 5' to 3' direction.

A "sequence coding for angiostatin" is a nucleic acid sequence which encodes the human angiostatin as described

above, based on the normal base pairing and translational codon usage relationships. The sequence coding for endostatin of similarly defined.

In a preferred embodiment the sequence coding for angiostatin is 5' to the sequence coding for endostatin. Those skilled in the art will appreciate that when more than two anti-angiogenic coding sequences are utilized, the anti-angiogenic coding sequences may all be on a single transcription unit, that all may be on separate transcription units, or that any two coding sequences may be on one transcription unit and the other coding sequence on another transcription unit (in the case of three coding sequences).

The plasmid may also contain an intron having variable splicing, a first coding sequence, and a second coding sequence, wherein the first and second coding sequences include a sequence coding for any two different anti-angiogenic agents, preferably angiostatin and endostatin.

In a preferred embodiment, the plasmid also has: (a) a transcriptional control sequence transcriptionally linked with a first coding sequence and a second coding sequence; (b) a 5'-untranslated region; (c) an intron 5' to the first coding sequence; (d) an alternative splice site 3' to the first coding sequence and 5' to the second coding sequence; and (e) a 3'-untranslated region/poly(A) signal. The transcriptional control sequence preferably includes a cytomegalovirus promoter sequence.

In a preferred embodiment, the plasmid also has: (a) a transcriptional control sequence transcriptionally linked with a first coding sequence, an IRES sequence, a second coding sequence, and a 3'-untranslated region/poly(A) signal, wherein the IRES sequence is between the first coding sequence and the second coding sequence; and (b) an intron between the promoter and the first coding sequence; wherein the first and second coding sequences include a sequence coding for any two different anti-angiogenic agents, preferably angiostatin and endostatin. The transcriptional control sequence preferably

includes a cytomegalovirus promoter sequence and the IRES sequence preferably is from an encephalomyocarditis virus.

For delivery of coding sequences for gene expression, it is generally useful to provide a delivery composition or delivery system which includes one or more other components in addition to the nucleic acid sequences. Such a composition can, for example, aid in maintaining the integrity of the DNA and/or in enhancing cellular uptake of the DNA and/or by acting as an immunogenic enhancer, such as by the non-DNA components having an immuno-stimulatory effect of their own.

Thus, in another aspect, the invention features a composition containing a plasmid as described above and a protective, interactive non-condensing compound (PINC).

The PINC enhances the delivery of the nucleic acid molecule to mammalian cells *in vivo*, and preferably the nucleic acid molecule includes a coding sequence for a gene product to be expressed in the cell. In many cases, the relevant gene product is a polypeptide or protein. Preferably the PINC is used under conditions so that the PINC does not form a gel, or so that no gel form is present at the time of administration at about 30-40°C. Thus, in these compositions, the PINC is present at a concentration of 30% (w/v) or less. In certain preferred embodiments, the PINC concentration is still less, for example, 20% or less, 10% or less, 5% or less, or 1% or less. Thus, these compositions differ in compound concentration and functional effect from uses of these or similar compounds in which the compounds are used at higher concentrations, for example in the ethylene glycol mediated transfection of plant protoplasts, or the formation of gels for drug or nucleic acid delivery. In general, the PINCs are not in gel form in the conditions in which they are used as PINCs, though certain of the compounds may form gels under some conditions.

In connection with the compounds and compositions of this invention, the term "protects" or "protective" refers to an effect of the interaction between such a compound and a

nucleic acid such that the rate of degradation of the nucleic acid is decreased in a particular environment. Such degradation may be due to a variety of different factors, which specifically include the enzymatic action of a nuclease. The protective action may be provided in different ways, for example, by exclusion of the nuclease molecules or by exclusion of water.

Some compounds which protect a nucleic acid and/or prolong the bioavailability of a nucleic acid may also interact or associate with the nucleic acid by intermolecular forces and/or valence bonds such as: Van der Waals forces, ion-dipole interactions, ion-induced dipole interactions, hydrogen bonds, or ionic bonds. These interactions may serve the following functions: (1) Stereoselectively protect nucleic acids from nucleases by shielding; (2) facilitate the cellular uptake of nucleic acid by "piggyback endocytosis". Piggyback endocytosis is the cellular uptake of a drug or other molecule complexed to a carrier that may be taken up by endocytosis. CV Uglea and C Dumitriu-Medvichi, *Medical Applications of Synthetic Oligomers*, In: Polymeric Biomaterials, Severian Dumitriu ed., Marcel Dekker, Inc., 1993, incorporated herein by reference.

To achieve the desired effects set forth it is desirable, but not necessary, that the compounds which protect the nucleic acid and/or prolong the bioavailability of a nucleic acid have amphiphilic properties; that is, have both hydrophilic and hydrophobic regions. The hydrophilic region of the compounds may associate with the largely ionic and hydrophilic regions of the nucleic acid, while the hydrophobic region of the compounds may act to retard diffusion of nucleic acid and to protect nucleic acid from nucleases.

Additionally, the hydrophobic region may specifically interact with cell membranes, possibly facilitating endocytosis of the compound and thereby also of nucleic acid associated with the compound. This process may increase the pericellular concentration of nucleic acid.

Agents which may have amphiphilic properties and are generally regarded as being pharmaceutically acceptable are the following: polyvinylpyrrolidones; polyvinylalcohols; polyvinylacetates; propylene glycol; polyethylene glycols; poloxamers (Pluronics); poloxamines (Tetronics); ethylene vinyl acetates; methylcelluloses, hydroxypropylcelluloses, hydroxypropylmethylcelluloses; heteropolysaccharides (pectins); chitosans; phosphatidylcholines (lecithins); miglyols; polylactic acid; polyhydroxybutyric acid; xanthan gum. Also, copolymer systems such as polyethylene glycol-polylactic acid (PEG-PLA), polyethylene glycol-polyhydroxybutyric acid (PEG-PHB), polyvinylpyrrolidone-polyvinylalcohol (PVP-PVA), and derivatized copolymers such as copolymers of N-vinyl purine (or pyrimidine) derivatives and N-vinylpyrrolidone. However, not all of the above compounds are protective, interactive, non-condensing compounds as described below.

In connection with the protective, interactive, non-condensing compounds for these compositions, the term "non-condensing" means that an associated nucleic acid is not condensed or collapsed by the interaction with the PINC at the concentrations used in the compositions. Thus, the PINCs differ in type and/or use concentration from such condensing polymers. Examples of commonly used condensing polymers include polylysine, and cascade polymers (spherical polycations).

Also in connection with such compounds and an associated nucleic acid molecule, the term "enhances the delivery" means that at least in conditions such that the amounts of PINC and nucleic acid is optimized, a greater biological effect is obtained than with the delivery of nucleic acid in saline. Thus, in cases where the expression of a gene product encoded by the nucleic acid is desired, the level of expression obtained with the PINC:nucleic acid composition is greater than the expression obtained with the same quantity of nucleic

acid in saline for delivery by a method appropriate for the particular PINC/coding sequence combination.

In preferred embodiments of the above compositions, the PINC is polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), a PVP-PVA co-polymer, N-methyl-2-pyrrolidone (NM2P), ethylene glycol, or propylene glycol. In compositions in which a Poloxamer (Pluronics) is used, the nucleic acid is preferably not a viral vector, i.e., the nucleic acid is a non-viral vector.

In other preferred embodiments, the PINC is bound with a targeting ligand. Such targeting ligands can be of a variety of different types, including but not limited to galactosyl, residues, fucosal residues, mannosyl residues, carntitine derivatives, monoclonal antibodies, polyclonal antibodies, peptide ligands, and DNA-binding proteins. The targeting ligands may bind with receptors on cells such as antigen-presenting cells, hepatocytes, myocytes, epithelial cells, endothelial cells, and cancer cells.

In connection with the association of a targeting ligand and a PINC, the term "bound with" means that the parts have an interaction with each other such that the physical association is thermodynamically favored, representing at least a local minimum in the free energy function for that association. Such interaction may involve covalent binding, or non-covalent interactions such as ionic, hydrogen bonding, van der Waals interactions, hydrophobic interactions, and combinations of such interactions.

While the targeting ligand may be of various types, in one embodiment the ligand is an antibody. Both monoclonal antibodies and polyclonal antibodies may be utilized.

The nucleic acid may also be present in various forms. Preferably the nucleic acid is not associated with a compounds(s) which alter the physical form, however, in other embodiments the nucleic acid is condensed (such as with a condensing polymer), formulated with cationic lipids,

formulated with peptides, or formulated with cationic polymers.

In preferred embodiments, the protective, interactive non-condensing compound is polyvinyl pyrrolidone, and/or the plasmid is in a solution having between 0.5% and 50% PVP, more preferably about 5% PVP. The DNA preferably is at least about 80% supercoiled, more preferably at least about 90% supercoiled, and most preferably at least about 95% supercoiled.

In another aspect the invention features a composition containing a protective, interactive non-condensing compound and a plasmid containing an anti-angiogenic coding sequence.

In yet another aspect, the invention provides a composition containing a plasmid of the invention (or a plasmid containing an anti-angiogenic coding sequence) and a cationic lipid, preferably with a neutral co-lipid.

Preferably the cationic lipid is DOTMA and the neutral co-lipid is cholesterol (chol). DOTMA is 1,2-di-O-octadecenyl-3-trimethylammonium propane, which is described and discussed in Eppstein et al., U.S. Patent 4,897,355, issued January 30, 1990, which is incorporated herein by reference. However, other lipids and lipid combinations may be used in other embodiments. A variety of such lipids are described in Gao & Huang, 1995, *Gene Therapy* 2:710-722, which is hereby incorporated by reference. Other cationic lipid delivery technology is described in Brigham, U.S. Patent 5,676,954, issued October 14, 1997, incorporated herein by reference in its entirety, including any drawings.

As the charge ratio of the cationic lipid and the DNA is also a significant factor, in preferred embodiments the DNA and the cationic lipid are present is such amounts that the negative to positive charge ratio is between 1:0.1 and 1:10, preferably between 1:0.3 and 1:6, more preferably about 1:3. While preferable, it is not necessary that the ratio be 1:3. Thus, preferably the charge ratio for the compositions is

between about 1:0.1 and 1:10, more preferably between about 1:0.3 and 1:6.

The term "cationic lipid" refers to a lipid which has a net positive charge at physiological pH, and preferably carries no negative charges at such pH. An example of such a lipid is DOTMA. Similarly, "neutral co-lipid" refers to a lipid which has is usually uncharged at physiological pH. An example of such a lipid is cholesterol.

Thus, "negative to positive charge ratio" for the DNA and cationic lipid refers to the ratio between the net negative charges on the DNA compared to the net positive charges on the cationic lipid.

As the form of the DNA affects the expression efficiency, the DNA preferably is at least about 80% supercoiled, more preferably at least about 90% supercoiled, and most preferably at least about 95% supercoiled. The composition preferably includes an isotonic carbohydrate solution, such as an isotonic carbohydrate solution that consists essentially of about 10% lactose. In preferred embodiments, the composition the cationic lipid and the neutral co-lipid are prepared as a liposome having an extrusion size of between 100 and 1,000 nanometers, preferably between 200 and 900 nanometers, more preferably about 800 nanometers. Preferably the liposomes are prepared to have an average diameter of between about 20 and 800 nm, more preferably between about 50 and 400 nm, still more preferably between about 75 and 200 nm, and most preferably about 100 nm. Microfluidization is the preferred method of preparation of the liposomes.

In another aspect the invention features a composition containing: (a) a first component having a plasmid including an anti-angiogenic coding sequence and a cationic lipid, preferably with a neutral co-lipid, wherein the cationic lipid is DOTMA and the neutral co-lipid is cholesterol, wherein the DNA in the plasmid and the cationic lipid are present in amounts such that the negative to positive charge ratio is between 1:0.1 and 1:10, preferably between 1:0.3 and 1:6, more

preferably about 1:3; and (b) a second component including a protective, interactive non-condensing compound, wherein the first component is present within the second component.

In another aspect, the invention provides a composition having a protective, interactive non-condensing compound, a first plasmid including a first anti-angiogenic coding sequence, preferably an angiostatin coding sequence and one or more other plasmids independently having a second anti-angiogenic subunit coding sequence, preferably an endostatin coding sequence.

In other aspects, the invention features a composition comprising a protective, interactive non-condensing compound, a first plasmid having an angiostatin coding sequence, and one or more other plasmids having an endostatin coding sequence.

Also provided by the present invention is a composition containing a plasmid with an anti-angiogenic coding sequence and a cationic lipid, preferably with a neutral co-lipid.

In another aspect, the invention features a method for making any of the plasmids described above by inserting a tissue specific element and an anti-angiogenic coding sequence into a plasmid.

The invention also provides methods of making the compositions described above. The method may involve: (a) preparing a DNA molecule having a transcriptional unit, wherein the transcriptional unit contains an anti-angiogenic coding sequence; (b) preparing a protective, interactive non-condensing compound; and (c) combining the protective, interactive non-condensing compound with the DNA in conditions such that a composition capable of delivering a therapeutically effective amount of an anti-angiogenic coding sequence to a mammal is formed.

Preferably, the DNA molecule is a plasmid, wherein the plasmid includes an anti-angiogenic coding sequence, and more preferably also includes a human growth hormone 3'-untranslated region/poly(A) signal.

The method may involve the steps of: (a) preparing a DNA having an anti-angiogenic coding sequence; (b) preparing a cationic lipid, preferably in a mixture with a neutral co-lipid, wherein the cationic lipid is DOTMA and the neutral co-lipid is cholesterol; and (c) combining the cationic lipid with the DNA in amounts such that the cationic lipid and the DNA are present in a negative to positive charge ratio between 1:0.1 and 1:10, preferably between 1:0.3 and 1:6, more preferably about 1:3.

In another embodiment, the method involves the steps of: (a) preparing a first component having a plasmid containing an anti-angiogenic coding sequence and a cationic lipid, preferably with a neutral co-lipid, wherein the cationic lipid is DOTMA and the neutral co-lipid is cholesterol, wherein the DNA in the plasmid and the cationic lipid are present in amounts such that the negative to positive charge ratio is between 1:0.1 and 1:10, preferably between 1:0.3 and 1:6, more preferably about 1:3; (b) preparing a second component having a protective, interactive non-condensing compound; and (c) combining the first and second components such that the resulting composition includes the first component within the second component.

In another embodiment, the method involves the steps of: (a) preparing a protective, interactive non-condensing compound, (b) preparing a first plasmid having a first anti-angiogenic coding sequence, preferably an angiostatin coding sequence (c) preparing one or more other plasmids independently having other anti-angiogenic coding sequence, preferably an endostatin coding sequence and (d) combining the protective, interactive non-condensing compound, the plasmid having the first anti-angiogenic coding sequence and the other plasmids.

The method of making a composition of the invention may also involve combining a plasmid with an anti-angiogenic coding sequence and a cationic lipid, and preferably also with a neutral co-lipid.

In another aspect, the invention provides a method for treatment of a mammalian condition or disease, by administering to a mammal suffering from the condition or disease a therapeutically effective amount of a plasmid as described herein.

A "therapeutically effective amount" of a composition is an amount which is sufficient to cause at least temporary relief or improvement in a symptom or indication of a disease or condition. Thus, the amount is also sufficient to cause a pharmacological effect. The amount of the composition need not cause permanent improvement or improvement of all symptoms or indications. A therapeutically effective amount of a cancer therapeutic would be one that reduces overall tumor burden in the case of metastatic disease (i.e., the number of metasteses or their size) or one that reduces the mass of the tumor in localized cancers.

The condition or disease preferably is a cancer, such as epithelial glandular cancer, including adenoma and adenocarcinoma; squamous and transitional cancer, including polyp, papilloma, squamous cell and transitional cell carcinoma; connective tissue cancer, including tissue type positive, sarcoma and other (oma's); hematopoietic and lymphoreticular cancer, including lymphoma, leukemia and Hodgkin's disease; neural tissue cancer, including neuroma, sarcoma, neurofibroma and blastoma; mixed tissues of origin cancer, including teratoma and teratocarcinoma. Other cancerous conditions that are applicable to treatment include cancer of any of the following: adrenal gland, anus, bile duct, bladder, brain tumors: adult, breast, cancer of an unknown primary site, carcinoids of the gastrointestinal tract, cervix, childhood cancers, colon and rectum, esophagus, gall bladder, head and neck, islet cell and other pancreatic carcinomas, Kaposi's sarcoma, kidney, leukemia, liver, lung: non-small cell, lung: small cell, lymphoma: AIDS-associated, lymphoma: Hodgkin's disease, Lymphomas: non-Hodgkin's disease, melanoma, mesothelioma, metastatic cancer, multiple myeloma,

ovary, ovarian germ cell tumors, pancreas, parathyroid, penis, pituitary, prostate, sarcomas of bone and soft tissue, skin, small intestine, stomach, testis, thymus, thyroid, trophoblastic disease, uterus: endometrial carcinoma, uterus: uterine sarcomas, vagina, or vulva. The composition preferably is administered by injection, although the method may also be performed *ex vivo*.

In another aspect, the invention provides a method for transfection (i.e., the delivery and expression of a gene to cells) of a cell *in situ*, by contacting the cell with a plasmid described herein for sufficient time to transfect the cell. Transfection of the cell preferably is performed *in vivo* and the contacting preferably is performed in the presence of about 5% PVP solution.

In another aspect, the invention features a method for delivery and expression of an anti-angiogenic gene in a plurality of cells, by: (a) transfecting the plurality of cells with a plasmid or composition of the invention; and (b) incubating the plurality of cells under conditions allowing expression of a nucleic acid sequence in the vector, wherein the nucleic acid sequence encodes an anti-angiogenic agent.

In preferred embodiments, the anti-angiogenic agent is a human anti-angiogenic agent and the cells are human cells and/or the contacting is performed in the presence of an about 5% PVP solution.

In another aspect, the invention features a method for treating a disease or condition, by transfecting a cell *in situ* with a plasmid or composition of the invention. By "*in situ*" is meant at the cell's naturally occurring location, which may be *in vivo* or *in vitro* depending upon the cell. Thus, as sued herein, *in situ* transfection, for example, is a term used primarily to distinguish from *ex vivo* transfection. The disease or condition can be a localized disease or condition (e.g., a solid tumor) or a systemic disease or condition. (e.g., a metastatic cancer).

In another aspect, the invention features a cell transfected with a plasmid or composition of the invention.

In yet another aspect, the invention features a method for treatment of a mammalian condition or disease, by administering to a mammal suffering from the condition or disease a therapeutically effective amount of a composition described herein.

A method for treatment of a mammalian condition or disease is also featured and involves administering to a mammal suffering from the condition or disease a therapeutically effective amount of a composition of a first plasmid with an angiostatin coding sequence and a second plasmid with an endostatin coding sequence.

As the compositions are useful for delivery of a nucleic acid molecule to cells *in vivo*, in a related aspect the invention provides a composition at an *in vivo* site of administration. In particular this includes at an *in vivo* site in a mammal.

In preferred embodiments the nucleic acid molecule includes a sequence encoding a gene product. Also in preferred embodiments, the site of administration is in an interstitial space or a tissue of an animal, particularly of a mammal.

The invention also provides methods for using the above compositions. Therefore, in further related aspects, methods of administering the compositions are provided in which the composition is introduced into a mammal, preferably into a tissue or an interstitial space.

Various methods of delivery may be utilized, such as are known in the art, but in preferred embodiments, the composition is introduced into the tissue or interstitial space by injection. The compositions may also be delivered to a variety of different tissues, but in preferred embodiments the tissue is muscle or tumor.

In another related aspect, the invention provides methods for treating a mammalian condition or disease by administering

a therapeutically effective amount of a composition as described above. In preferred embodiments, the disease or condition is a cancer.

The summary of the invention described above is non-limiting and other features and advantages of the invention will be apparent from the following detailed description of the preferred embodiments, as well as from the claims.

Brief Description Of The Drawings

Figure 1 shows plasmid maps for pES1100, pES1062, and pES1281.

Figure 2 shows plasmid maps for pAS1095 and pAS1096.

Figure 3 shows plasmid information for various endothelial cell-specific constructs.

Figure 4 shows a plasmid map for pLC1264.

Figure 5 shows luciferase activity and endothelial cell specificity of plasmids of the invention.

Figure 6 shows a procedure for multimerization of an endothelial enhancer.

Figure 7 shows plasmid maps for pAS1359 and pES1358.

Figure 8 shows *in vitro* expression of bioactive endostatin.

Figure 9 shows that endostatin/PVP inhibits Renca tumor.

Figure 10 shows that endostatin/PVP induces apoptosis of EC.

Figure 11 shows endostatin and angiostatin expresion in serum after intramuscular delivery.

Figure 12 shows that endostatin/PVP inhibits sc Renca tumor after im delivery.

Figure 13 shows that endostatin/PVP inhibits sc Renca tumor after im delivery.

Figure 14 shows endostatin transgene mRNA in lung.

Figure 15 shows a mouse cornea angiogenesis assay.

Figure 16 shows a preferred codon usage table.

Detailed Description Of The Preferred Embodiments

The plasmids, related products and methods of the invention are described in detail below.

I. General

This invention concerns expression systems for the delivery and expression of anti-angiogenic coding sequences in mammalian cells, and formulations and methods for delivering such expression systems or other expression systems to a mammal.

Therefore, particular genetic constructs are described which include nucleotide sequences coding for anti-angiogenic agents, preferably human endostatin or angiostatin. Such a construct can beneficially be formulated and administered as described herein, utilizing the expression systems of this invention.

To allow convenient production of such plasmids, it is generally preferable that the plasmid be capable of replication in a cell to high copy number. Generally such production is carried out in prokaryotic cells, particularly including *Esherichia coli* (*E.coli*) cells. Thus, the plasmid preferably contains a replication origin functional in a prokaryotic cell, and preferably the replication origin is one which will direct replication to a high copy number.

It is also possible to utilize synthetic genetic elements in the plasmid constructs.

As described below, these elements affect post-transcriptional processing in eukaryotic systems. Thus, the use of synthetic sequences allows the design of processing characteristics as desired for the particular application. Commonly, the elements will be designed to provide rapid and accurate processing.

For delivery of DNA encoding a desired expression product to a mammalian system, it is usually preferable to utilize a delivery system. Such a system can provide multiple benefits,

notably providing stabilization to protect the integrity of the DNA, as well as assisting in cellular uptake.

In addition, the non-DNA components of the formulation may contribute to an immune system enhancement or activation. As a result, components of a delivery system can be selected in conjunction with a particular gene product to enhance or minimize the immuno-stimulatory effect.

The plasmids may also include elements for expression of an anti-cancer or anti-tumor agent, such as cytokine, for example IL-12 or one or more subunits thereof.

A "subunit" of a therapeutic molecule is a polypeptide or RNA molecule which combines with one or more other molecules to form a complex having the relevant pharmacologic activity. Examples of such complexes include homodimers and heterodimers as well as complexes having greater numbers of subunits. A specific example of a heterodimer is IL-12, having the p40 and p35 subunits.

Similarly, the treatment may involve administration of an anti-angiogenic coding sequence and one or more cytokine or other anti-cancer or anti-tumor coding sequences whether on a single plasmid or on separate plasmids. Such plasmids may be incorporated into compositions for delivery with a protective, interactive non-condensing compound, a cationic lipid and neutral co-lipid, or both.

While these are specific effective examples, other components may be utilized in formulations containing the anti-angiogenic expression vectors of the present invention to provide effective delivery and expression of anti-angiogenic agents or with other coding sequences for which manipulation of the activation of immune system components is desirable.

The selection of delivery system components and preparation methods in conjunction with the selection of coding sequences provides the ability to balance the specific effects of the encoded gene products and the immune system effects of the overall delivery system composition. This capacity to control the biological effects of delivery system

formulation administration represents an aspect of the invention in addition to the anti-angiogenic agent encoding constructs and specific formulations of delivery systems. Co-selection of the encoded gene product and the delivery system components and parameters provides enhanced desired effects rather than merely providing high level gene expression. In particular, such enhancement is described below for the antitumor effects of the exemplary PVP containing compositions.

For systems in which IL-12 is also administered, the antitumor effect may be greater than merely additive (i.e., greater than merely the sum of the antitumor effects of the anti-angiogenic agent alone and IL-12 alone). Enhancement of immuno-stimulatory effects is also desirable in other contexts, for example, for vaccine applications.

In contrast, for certain applications, it is preferable to select a delivery systems which minimizes the immune system effects. For example, it is often preferred that the immune system activation be minimized for compositions to be delivered to the lung in order to minimize lung tissue swelling.

A useful approach for selecting the delivery system components and preparation techniques for a particular coding sequence can proceed as follows, but is not limited to these steps or step order.

1. Select a particular genetic construct which provides appropriate expression *in vitro*.

2. Select delivery system components based on desired immunostimulatory effects and/or on *in vivo* physiological effect. Such effects can be tested or verified in *in vivo* model systems.

3. Select the other delivery system parameters consistent with the desired immuno-stimulatory effects and/or consistent with enhancing the desired *in vivo* physiological effect. Such parameters can, for example, include the amount and ratio of DNA to one or more other composition components,

the relative amounts of non-DNA composition components, the size of delivery system formulation particles, the percent supercoiled DNA for circular dsDNA vectors, and the specific method of preparation of delivery system formulation particles. The particular parameters relevant for specific types of formulations will be apparent or readily determined by testing.

The description below illustrates the selection of components and parameters in the context of anti-angiogenic agent encoding constructs. However, it should be recognized that the approach is applicable to constructs containing a variety of other coding sequences.

II. Plasmid Construct Expression Systems

A. Plasmid Design and Construction

For the methods and constructs of this invention, a number of different plasmids were constructed which are useful for delivery and expression of sequences encoding anti-angiogenic agents. Thus, these plasmids contain coding regions for anti-angiogenic agents, along with genetic elements necessary or useful for expression of those coding regions.

While these embodiments utilized cDNA clones or partial genomic sequences from a particular source, those skilled in the art could readily obtain anti-angiogenic coding sequences from other sources, or obtain a coding sequence by identifying a cDNA clone in a library using a probe(s) based on the published anti-angiogenic coding and/or flanking sequences.

Coding sequences for anti-angiogenic agents were incorporated into an expression plasmid that contains eukaryotic and bacterial genetic elements. Eukaryotic genetic elements include the CMV immediate early promoter and 5' UTR, and a human growth hormone 3' UTR/poly(a) signal, which influence gene expression by controlling the accuracy and efficiency of RNA processing, mRNA stability, and translation.

The human growth hormone 3' UTR is from a human growth hormone gene, and preferably includes a poly(a) signal. This sequence can be linked immediately following the natural translation termination codon for a cDNA sequence, genomic sequence, modified genomic sequence, or synthetic sequence coding for anti-angiogenic agent.

An example of a human growth hormone 3' UTR/poly(a) signal is shown by the human growth hormone 3' UTR (nucleotides 1 - 100) and 3' flanking sequence (nucleotides 101 - 191; GenBank accession #J03071, HUMGHCSA) below.

```
  1  GGGTGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGT
                                    Poly (a)signal
 51  TGCCACTCCAGTGCCCACCAGCCTTGTCCTAATAAAATTAAGTTGCATCA
101  TTTTGTCTGACTAGGTGTCCTTCTATAATATTATGGGGTGGAGGGGGGTG
151  GTATGGAGCAAGGGGCAAGTTGGGAAGACAACCTGTAGGGC
```

The 5' and 3' UTR and flanking regions can be further and more precisely defined by routine methodology, *e.g.*, deletion or mutation analysis or their equivalents., and can be modified to provide other sequences having appropriate transcriptional and translational functions. Construction of plasmid, plasmid backbone, human anti-angiogenic cDNA, and final construct is described below in the examples.

Several EC-specific promoters, discussed below, have been described in the literature.

The human Von Willebrand factor (vWF) gene flanking region and the first exon, shown to support high-level and EC-specific expression *in vitro*, expressed lacZ in only a sub-population of EC *in vivo* and not in the vascular beds of various organs examined (Aird et al., *PNAS* 92:4567-4571, (1995)). It is composed of a non-specific core promoter (-90 to +22), a negative element that inhibits its activity in all cell types (-478 to -300), and a positive element that relieves repression only in EC and results in EC-specific expression (+150 to +247). It has been shown that this positive element is not present on the bovine vWF promoter.

The preproendothelin (ET-1) gene promoter is a 119 base-pair ("bp") fragment of human Endothelin gene promoter (-240 to -86) which directs EC-specific expression of CAT when fused to minimal SV40 promoter. Murine ET-1 promoter directs expression of either LUC or lipid-peroxidating enzyme in transgenic mice (Harats et al., *JCI* 95:1335-44, 1995). However, expression of the transgenes was not confined to vascular EC, but also present in arteries smooth muscle, and selected epithium. Moreover, the level of expression ranged from high in arteries to low in veins and capillaries, and there was significant variation in expression both between and withing organs.

The StyI (-336 to +23) fragment of intracellular adhesion molecule-2 (ICAM-2) gene promoter has been shown to direct heterologous gene (CD59) expression to kidney and lung vasculature in trangenic mice. It is TATA-less promoter and contains Sp1, GATA and ETS binding sites.

Alpha v beta 3 integrin is preferentially expressed in tumor endothelium. In contrast to alpha v beta 3 integrin (fibronectin receptor), the alpha v beta 3 integrin (vitronectin receptor) cooperates with certain growth factors. Inhibition of its expression blocks new vessel formation during human would healing.

A 15.5 kb DNA fragment that contains the 5' flanking region, the first exon, and part of the first intron of human alpha v gene, was determined and named the Human Alpha v gene promoter. The transcription initiation site was mapped 169 bp upstream of ATG site. The 5'flanking region does not contain a TATA box or initiation element, but does contain four Sp1, two Ets and one GATA binding site. The 222 bp region of alpha v gene promoter has been shown to exert a strong positive effect on alpha v promoter activity.

A 6 kb human genomic DNA fragment containing 2.0 kb of the sequence 5' to the start codon is defined as the human beta 3 gene promoter. The 584-bp fragment 5' to the start codon promotes expression of the CAT reporter gene by 5-fold

over promoter-less control CAT construct. This beta 3 promoter lacks TATA and CAAT cis-acting elements, but there are two Sp1 sites flanking the transcription start site. It has been shown that beta 3 promoter can be upregulated by PMA and retinoic acid, but not by proinflammatory cytokine such as TNF/IFN-gamma.

Vascular endothelial growth factor receptor (VEGFR) and its two EC-specific receptor tyrosine kinases, Flk-1/KDR and Flt-1, play key roles in physiological and pathological angiogenesis. The -3118 to +209 fragment of the mouse Flt gene promoter and the -1829 to +148 fragment of mouse Flk-1 gene promoter have been cloned. Hypoxia has been shown to be a major mechanism for up-regulation of VEGF and its receptors *in vivo*. In transient transfection assays, hypoxia led to strong transcriptional activation of the Flt-1 promoter, whereas Flk-1/KDR transcription was essentially unchanged. A 430-bp region of the Flt-1 promoter is required for transcription in response to hypoxia and this region includes a hypoxia-inducible factor (HIF) consensus sequence.

The Endothelin-1 enhancer, an endothelial cell-specific regulatory region located between 320 and 364 bp upstream of the transcription initiation site of the mouse endothelin-1 gene, was identified by Bu and Quartermous (*J. Biol. Chem.* 272:32613-32622, (1997)). Three copies of this enhancer sequence have been shown to activate both the ET-1 promoter and heterologous promoters.

Gene expression driven by the cell-cycle-specific promoters cyclin A, E2F1, or cdc6 is regulated in a cell-cycle-dependent fashion and this regulation is primarily at the transcriptional level. The promoters of these genes contain common E2F sites which are responsible for repression in the resting G0 (zero) phase, and in some cases for activation in cycling cells. (Henglein, B., X. Chenivesse, J. Wang, D. Eick, and C. Brechot. 1994. The structure and cell cycle-regulated transcription of the human cyclin A gene is described in *Proc. Natl. Acad. Sci. USA,* 91:5490-5494.

Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression is described in Genes & Dev. 8:1514-1525; Williams, R. S., R. V. Shohet, and B. Stillman 1997. A human protein related to yeast Cdc6p expression is described in *Proc. Natl. Acad. Sci. USA* 94:142-147; Yan, Z., J. DeGregori, R. Shohet, G. Leone, B. Stillman, J. R. Nevins, and R. S. Williams, 1998. Cdc6 is regulated by E2F and is essential for DNA replication in mammalian cells.

B. Synthetic Genetic Elements

In some embodiments, some or all of the genetic elements can be synthetic, derived from synthetic oligonucleotides, and thus are not obtained directly from natural genetic sequences. These synthetic elements are appropriate for use in many different expression vectors.

A synthetic intron is designed with splice sites that ensure that RNA splicing is accurate and efficient. A synthetic 3' UTR/poly(A) signal is designed to facilitate mRNA 3' end formation and mRNA stability. A synthetic 5' UTR is designed to facilitate the initiation of translation. The design of exemplary synthetic elements is described in more detail below.

Summary of Synthetic Element Features

Exemplary synthetic 5'UTR, intron, and 3'UTR/poly(A) signal have the general features shown below:

5' UTR
- Short.
- Lack of secondary structure.
- Kozak sequence.
- Site for intron insertion.

Intron	5' splice site sequence matches consensus. 5' splice site sequence is exactly complementary to 5' end of U1 snRNA. Branch point sequence matches consensus. Branch point sequence is complementary to U2 snRNA. 3' splice site matches consensus. Polypyrimidine tract is 16 bases in length and contains 7 consecutive T's. (The tract preferably contains at least 5 consecutive T's.) Contains internal restriction enzyme sites. BbsI cleaves at the 5'ss, EarI cleaves at the 3'ss.
3' UTR/Poly(A)	Based on rabbit β-globin 3' UTR/poly(A) signal. Consists of two poly(A) signals in tandem.

Features of the Synthetic 5'UTR (UT6):

The 5' untranslated region (5'UTR) influences the translational efficiency of messenger RNA, and is therefore an important determinant of eukaryotic gene expression. The synthetic 5'UTR sequence (UT6) has been designed to maximize the translational efficiency of mRNAs encoded by vectors that express genes of therapeutic interest.

The sequence of the synthetic 5' UTR (UT6) is shown below. The Kozak sequence is in boldface and the initiation codon is double underlined. The location of the intron (between residues 48 and 49) is indicated by the triangle and the sequences that form the exonic portion of consensus splice sites are single underlined. The restriction sites for HindIII and NcoI are overlined.

HindIII ∇ NcoI

AAGCTTACTCAACACAATAACAAACTTACTTACAATCTTAATTAACAGG**CCACCATGG**

The 5' untranslated region (5' UTR), located between the cap site and initiation codon, is known to influence the efficiency of mRNA translation. Any features that influence

the accessibility of the 5' cap structure to initiation factors, the binding and subsequent migration of the 43S preinitiation complex, or the recognition of the initiation codon, will influence mRNA translatability. An efficient 5' UTR is expected to be one that is moderate in length, devoid of secondary structure, devoid of upstream initiation codons, and has an AUG within an optimal local context (Kozak, 1994, *Biochimie* 76:815-821; Jansen et al., 1994). A 5' UTR with these characteristics should allow efficient recognition of the 5' cap structure, followed by rapid and unimpeded ribosome scanning by the ribosome, thereby facilitating the translation initiation process.

The sequence of the synthetic 5'UTR was designed to be moderate in length (54 nucleotides (nts)), to be deficient in G but rich in C and A residues, to lack an upstream ATG, to place the intended ATG within the context of a optimal Kozak sequence (CCACCATGG), and to lack potential secondary structure. The synthetic 5' UTR sequence was also designed to lack AU-rich sequences that target mRNAs to be rapidly degraded in the cytoplasm.

Experiments have demonstrated that introns increase gene expression from cDNA vectors, and that introns located in the 5' UTR are more effective than ones located in the 3' UTR (Huang and Gorman, 1990, *Mol. Cell. Biol.* 10:1805-1810; Evans and Scarpulla, 1989, *Gene* 84:135-142; Brinster et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:836-840; Palmiter et al., 1991, *Proc. Natl. Acad. Sci. USA* 88:478-482; Choi et al., 1991, *Mol. Cell. Biol.* 11:3070-3074). Accordingly, the synthetic 5' UTR sequence was designed to accommodate an intron with consensus splice site sequences. The intron may, for example, be located between residues 48 and 49 (See intron sequence structure below). The CAG at position 46-48 is the exonic portion of a consensus 5' splice site. The G at position 49 is the exonic portion of a consensus 3' splice site.

To facilitate cloning manipulations, the synthetic 5' UTR sequence was designed to begin with a HindIII site and terminate with a NcoI site.

Features of the Synthetic Intron

RNA splicing is required for the expression of most eukaryotic genes. For optimal gene expression, RNA splicing must be highly efficient and accurate. A synthetic intron, termed OPTIVS8B, was designed to be maximally efficient and accurate.

The structure of the exemplary synthetic intron, OPTIVS8 is shown below. Sequences for the 5' splice site (5'ss), branch point (bp), and 3' splice site (3'ss) are double underlined. The recognition sequences for the restriction enzymes BbsI and EarI are overlined. The cleavage site for BbsI corresponds to the 5'ss, and the cleavage site for EarI corresponds to the 3'ss.

```
5'ss                          bp                          3'ss
 |       BbsI                  |                EarI       |
5'CAG GTAAGTGTCTTC---(77)---TACTAACGGTTCTTTTTTCTCTTCACAG G 3'
```

The 5' splice site (5'ss) sequence matches the established consensus sequence, MAG ↓ GTRAGT, where M = C or A, and R = G or A. Since the mechanism of splicing involves an interaction between the 5'ss of the pre-mRNA and U1 snRNA, the 5'ss sequence of OPTIVS8B was chosen to be exactly complementary to the 5' end of U1 snRNA.

```
5'ss      5' CAGGUAAGU 3'
               |||||||||
U1 RNA    3' GUCCAUUCA 5'
```

In mammals, the consensus sequence for branch points (YNYTRAY, where Y = C or T, R = A or G, N = any base, and the underlined A residue is the actual branch point) is very ambiguous. Since the mechanism of splicing involves an interaction between the branch point (bp) of the pre-mRNA and U2 snRNA, the branch point sequence of OPTIVS8B was chosen to maximize this interaction. (Note that the branch point itself

is bulged out). The chosen sequence also matches the branch point sequence that is known to be obligatory for pre-mRNA splicing in yeast. The branch point is typically located 18-38 nucleotides (nts) upstream of the 3' splice site. In OPTIVS8B, the branch point is located 24 nts upstream from the 3' splice site.

```
BP          5' UACUAAC 3'
                 ||||| |
U2 RNA      3' AUGAU G 5'
```

The sequence of the 3' splice site (3'ss) matches the established consensus sequence, Y_{11}NYAG ↓ G, where Y = C or T, and N = any base. In 3' splice sites, the polypyrimidine tract (Y_{11}) is the major determinant of splice site strength. For optimal splice site function in OPTIVS8B, the length of the polypyrimidine tract was extended to 16 bases, and its sequence was adjusted to contain 7 consecutive T residues. This feature was included because optimal splicing requires the presence of at least 5 consecutive T residues in the polypyrimidine tract.

Splicing *in vitro* is generally optimal when introns are >80 nts in length (Wieringa, et al., 1984; Ulfendahl et al., 1985, *Nucl. Acids Res.* 13:6299-6315). Although many introns may be thousands of bases in length, most naturally occurring introns are 90-200 nt in length (Hawkins, 1988, *Nucl. Acids Res.* 16:9893-9908). The length of the synthetic intron (118 nts) falls within this latter range.

OPTIVS8B was designed with three internal restriction enzyme sites, BbsI, NheI, and EarI. These restriction sites facilitate the screening and identification of genes that contain the synthetic intron sequence. In addition, the BbsI and EarI sites were placed so that their cleavage sites exactly correspond to the 5'ss (BbsI) or 3'ss (EarI). The sequence of the polypyrimidine tract was specifically designed to accommodate the EarI restriction site. Inclusion of the BbsI and EarI sites at these locations is useful because they permit the intron to be precisely deleted from a gene. They

also permit the generation of an "intron cassette" that can be inserted at other locations within a gene.

The 77 bases between the BbsI site and the branch point sequence are random in sequence, except for the inclusion of the NheI restriction site.

Features of the Synthetic 3' UTR/poly(A) Signal:

The 3' ends of eukaryotic mRNAs are formed by the process of polyadenylation. This process involves site specific site RNA cleavage, followed by addition of a poly(A) tail. RNAs that lack a poly(A) tail are highly unstable. Thus, the efficiency of cleavage/polyadenylation is a major determinant of mRNA levels, and thereby, of gene expression levels. 2XPA1 is a synthetic sequence, containing two efficient poly(A) signals, that is designed to be maximally effective in polyadenylation.

A poly(A) signal is required for the formation of the 3' end of most eukaryotic mRNA. The signal directs two RNA processing reactions: site-specific endonucleolytic cleavage of the RNA transcript, and stepwise addition of adenylates (approximately 250) to the newly generated 3' end to form the poly(A) tail. A poly(A) signal has three parts: hexanucleotide, cleavage site, and downstream element. The hexanucleotide is typically AAUAAA and cleavage sites are most frequently 3' to the dinucleotide CA (Sheets et al., 1987). Downstream elements are required for optimal poly(A) signal function and are located downstream of the cleavage site. The sequence requirement for downstream elements is not yet fully established, but is generally viewed as UG- or U-rich sequences (Wickens, 1990; Proudfoot, 1991, *Cell* 64:671-674; Wahle, 1992, *Bioessays* 14:113-118; Chen and Nordstrom, 1992, *Nucl. Acids Res.* 20:2565-2572).

Naturally occurring poly(A) signals are highly variable in their effectiveness (Peterson, 1992). The effectiveness of a particular poly(A) signal is mostly determined by the quality of the downstream element. (Wahle, 1992). In

expression vectors designed to express genes of therapeutic interest, it is important to have a poly(A) signal that is as efficient as possible.

Poly(A) efficiency is important for gene expression, because transcripts that fail to be cleaved and polyadenylated are rapidly degraded in the nuclear compartment. In fact, the efficiency of polyadenylation in living cells is difficult to measure, since nonpolyadenylated RNAs are so unstable. In addition to being required for mRNA stability, poly(A) tails contribute to the translatability of mRNA, and may influence other RNA processing reactions such as splicing or RNA transport (Jackson and Standart,1990, *Cell* 62:15-24; Wahle, 1992).

Some eukaryotic genes have more than one poly(A) site, implying that if the cleavage/polyadenylation reaction fails to occur at the first site, it will occur at one of the later sites. In COS cell transfection experiments, a gene with two strong poly(A) sites yielded approximately two-fold more mRNA than one with a single strong poly(A) site (Bordonaro, 1995). These data suggest that a significant fraction of transcripts remain unprocessed even with a single "efficient" poly(A) signal. Thus, it may be preferable to include more than one poly(A) site.

The sequence of the exemplary synthetic poly(A) signal is shown below. The sequence is named 2XPA. The hexanucleotide sequences and downstream element sequences are double underlined, and the two poly(A) sites are labeled as pA#1 and pA#2. Convenient restriction sites are overlined. The entire 2XPA unit may be transferred in cloning experiments as a XbaI-KpnI fragment. Deletion of the internal BspHI fragment results in the formation of a 1XPA unit.

XbaI BspHI

TCTAGAGCATTTTTCCCTCTGCCAAAAATTATGGGGACATCATGAAGCCCCTTGAGCATCTGAC

G

pA#1

Hex | Downstream element

TCTGGCTAATAAAGGAAATTTATTTTCATTGCAATAGTGTGTTGGAATTTTTTGTGTCTCTCAC

T

BspHI

CGGTACTAGAGCATTTTTCCCTCTGCCAAAAATTATGGGGACATCATGAAGCCCCTTGAGCATC

T

pA#2

Hex | Downstream element

GACGTCTGGCTAATAAAGGAAATTTATTTTCATTGCAATAGTGTGTTGGAATTTTTTGTGTCTC

T

KpnI

CACTCGGTACC

The sequence of the synthetic poly(A) site shown above is based on the sequence of the rabbit β-globin poly(A) signal, a signal that has been characterized in the literature as strong (Gil and Proudfoot, 1987, *Cell* 49:399-406; Gil and Proudfoot, 1984, *Nature* 312:473-474). One of its key features is the structure of its downstream element, which contains both UG- and U-rich domains.

A double-stranded DNA sequence corresponding to the 1XPA sequence was constructed from synthetic oligonucleotides. Two copies of the 1XPA sequence were then joined to form the 2XPA sequence. The sequences were joined in such as way as to have a unique XbaI site at the 5' end of the first poly(A) signal containing fragment, and a unique KpnI site at the 3' end of the second poly(A) signal containing fragment.

C. Coding Sequences

The nucleotide sequences of several natural human anti-angiogenic coding sequences are known, and are provided below, along with a synthetic sequence which also codes for an anti-angiogenic agent.

In some cases, instead of the natural sequence coding for the anti-angiogenic agent of interest, it is advantageous to utilize synthetic sequences which encode the anti-angiogenic agent. Such synthetic sequences have alternate codon usage from the natural sequence, and thus have dramatically different nucleotide sequences from the natural sequence. In particular, synthetic sequences can be used which have codon usage at least partially optimized for expression in a human. The natural sequences do not have such optimal codon usage. Preferably, substantially all the codons are optimized.

Optimal codon usage in humans is indicated by codon usage frequencies for highly expressed human genes, as shown in **Fig. 16**. The codon usage chart is from the program "Human_High.cod" from the Wisconsin Sequence Analysis Package, Version 8.1, Genetics Computer Group, Madison, WI. The codons which are most frequently used in highly expressed human genes are presumptively the optimal codons for expression in human host cells, and thus form the basis for constructing a synthetic coding sequence. An example of a synthetic anti-angiogenic coding sequence is shown as the bottom sequence in the table below.

However, rather than a sequence having fully optimized codon usage, it may be desirable to utilize a sequence which has optimized codon usage except in areas where the same amino acid is too close together or abundant to make uniform codon usage optimal.

In addition, other synthetic sequences can be used which have substantial portions of the codon usage optimized, for example, with at least 50%, 70%, 80% or 90% optimized codons as compared to a natural coding sequence. Other particular synthetic sequences can be selected by reference to the codon usage chart in **Fig. 16**. A sequence is selected by choosing a codon for each of the amino acids of the polypeptide sequences. DNA molecules corresponding to each of the polypeptides can then by constructed by routine chemical synthesis methods. For example, shorter oligonucleotides can

be synthesized, and then ligated in the appropriate relationships to construct the full-length coding sequences.

Those skilled in the art will realize that various nucleic acid sequences with optimized codon usage can be constructed.

The preferred codon usage for human IP-10, endostatin and angiostatin are set forth below.

Preferred codon sequence for human IP-10

```
  1 aagcttacca tgaaccagac cgccatcctg atctgctgcc tgatcttcct gaccctgagc
 61 ggcatccagg gcgtgccccc gagccgcacc gtgcgctgca cctgcatcag catcagcaac
121 cagcccgtga acccccgcag cctggagaag ctggagatca tccccgccag ccagttctgc
181 ccccgcgtgg agatcatcgc caccatgaag aagaagggcg agaagcgctg cctgaacccc
241 gagagcaagg ccatcaagaa cctgctgaag gccgtgagca aggagatgag caagcgcagc
301 ccgggcggag gtggcagcgg cggaggtggc agcggcggag gtggcagcgg atcctctaga
```

Preferred codon sequence for human Endostatin

```
  1 CACAGCCACC GCGACTTCCA GCCCGTGCTG CACCTGGTGG CCCTGAACAG
 51 CCCCCTGAGC GGCGGCATGC GCGGCATCCG CGGCGCCGAC TTCCAGTGCT
101 TCCAGCAGGC CCGCGCCGTG GGCCTGGCCG GCACCTTCCG CGCCTTCCTG
151 AGCAGCCGCC TGCAGGACCT GTACAGCATC GTGCGCCGCG CCGACCGCGC
201 CGCCGTGCCC ATCGTGAACC TGAAGGACGA GCTGCTGTTC CCCAGCTGGG
251 AGGCCCTGTT CAGCGGCAGC GAGGGCCCCC TGAAGCCCGG CGCCCGCATC
301 TTCAGCTTCG ACGGCAAGGA CGTGCTGCGC CACCCCACCT GGCCCCAGAA
351 GAGCGTGTGG CACGGCAGCG ACCCCAACGG CCGCCGCCTG ACCGAGAGCT
401 ACTGCGAGAC CTGGCGCACC GAGGCCCCCA GCGCCACCGG CCAGGCCAGC
451 AGCCTGCTGG GCGGCCGCCT GCTGGGCCAG AGCGCCGCCA GCTGCCACCA
501 CGCCTACATC GTGCTGTGCA TCGAGAACAG CTTCATGACC GCCAGCAAGT
551 GA
```

Preferred codon sequence for human Angiostatin

```
  1 ATGGAGCACA AGGAGGTGGT GCTGCTGCTG CTGCTGTTCC TGAAGAGCGG
 51 CCAGGGCGAG CCCCTGGACG ACTACGTGAA CACCCAGGGC GCCAGCCTGT
101 TCAGCGTGAC CAAGAAGCAG CTGGGCGCCG GCAGCATCGA GGAGTGCGCC
151 GCCAAGTGCG AGGAGGACGA GGAGTTCACC TGCCGCGCCT TCCAGTACCA
201 CAGCAAGGAG CAGCAGTGCG TGATCATGGC CGAGAACCGC AAGAGCAGCA
```

```
251  TCATCATCCG CATGCGCGAC GTGGTGCTGT TCGAGAAGAA GGTGTACCTG
301  AGCGAGTGCA AGACCGGCAA CGGCAAGAAC TACCGCGGCA CCATGAGCAA
351  GACCAAGAAC GGCATCACCT GCCAGAAGTG GAGCAGCACC AGCCCCCACC
401  GCCCCCGCTT CAGCCCCGCC ACCCACCCCA GCGAGGGCCT GGAGGAGAAC
451  TACTGCCGCA ACCCCGACAA CGACCCCCAG GGCCCCTGGT GCTACACCAC
501  CGACCCCGAG AAGCGCTACG ACTACTGCGA CATCCTGGAG TGCGAGGAGG
551  AGTGCATGCA CTGCAGCGGC GAGAACTACG ACGGCAAGAT CAGCAAGACC
601  ATGAGCGGCC TGGAGTGCCA GGCCTGGGAC AGCCAGAGCC CCCACGCCCA
651  CGGCTACATC CCCAGCAAGT TCCCCAACAA GAACCTGAAG AAGAACTACT
701  GCCGCAACCC CGACCGCGAG CTGCGCCCCT GGTGCTTCAC CACCGACCCC
751  AACAAGCGCT GGGAGCTGTG CGACATCCCC CGCTGCACCA CCCCCCCCCC
801  CAGCAGCGGC CCCACCTACC AGTGCCTGAA GGGCACCGGC GAGAACTACC
851  GCGGCAACGT GGCCGTGACC GTGAGCGGCC ACACCTGCCA GCACTGGAGC
901  GCCCAGACCC CCCACACCCA CAACCGCACC CCCGAGAACT TCCCCTGCAA
951  GAACCTGGAC GAGAACTACT GCCGCAACCC CGACGGCAAG CGCGCCCCCT
1001 GGTGCCACAC CACCAACAGC CAGGTGCGCT GGGAGTACTG CAAGATCCCC
1051 AGCTGCGACA GCAGCCCCGT GAGCACCGAG CAGCTGGCCC CCACCGCCCC
1101 CCCCGAGCTG ACCCCCGTGG TGCAGGACTG CTACCACGGC GACGGCCAGA
1151 GCTACCGCGG CACCAGCAGC ACCACCACCA CCGGCAAGAA GTGCCAGAGC
1201 TGGAGCAGCA TGACCCCCCA CCGCCACCAG AAGACCCCCG AGAACTACCC
1251 CAACGCCGGC CTGACCATGA ACTACTGCCG CAACCCCGAC GCCGACAAGG
1301 GCCCCTGGTG CTTCACCACC GACCCCAGCG TGCGCTGGGA GTACTGCAAC
1351 CTGAAGAAGT GC
```

Examples of various anti-angiogenic factors which can be encoded by the plasmids of the invention are described below. Additional examples are described in Mixson, European Patent Publication EP 0819 758 A2, published January 21, 1998, incorporated herein by reference in its entirety, including any drawings.

1. Endostatin, and Angiostatin:

Endostatin is a member of the expanding angiogenensis inhibitor family of proteins. It is a 20 KDa C-terminal fragment (184 a.a.) of collagen XVIII and selectively inhibits endothelial cell proliferation *in vitro* and angiogenesis *in vivo* (O'Reilly, M.S. et al., *Cell* **88**, 277-285, 1997). Angiostatin is an internal proteolytic fragment of mature

plasminogen (38 Kda and 362 a.a) (O'Reilly, M.S. et al., *Cell* **79**, 315-328, 1994). It contains four triple loop disulfide-linked structures, known as kringle domains. It has been shown that three kringle domain form is more potent (Cao, Y. et al., *J. Biol. Chem.*, **271**, 29461-7, 1996). *E. coli* expressed rEndostatin or Angiostatin was injected at high doses (10 mg/kg/d for 15-16 days) to achieve 97% inhibition of tumor growth efficacy in preclinical studies. It has been claimed that Angiostatin has a half-life of 2 days in blood.

2. Tumor suppressor genes:

p53 and its induced anti-angiogenic protein throbospondin-1 (TSP-1): p53 is mutated in half of human tumors. Wild-type p53 regulates cell cycle by through p21 (a inhibitor of cyclin-depedent) and pRB (another tumor suppressor). Also, p53 induces synthesis of the anti-angiogenic factor TSP-1, a large trimeric glycoprotein composed of three identical 180 kd subunits linked by disulfide bonds. TSPf, a fragment of TSP-1 encoded by 1013-1650 of TSP-1 gene, has more potent anti-angiogenic activity. Systemic intravenous administration of α-actin driven p53 gene complexed to cationic liposomes has been found reduce growth and metastases of a malignant human breast cancer in nude mice (Lesoon-Wood *et al.*, *PNAS*, 36:421, 1995). The primary target is thought to be the vasculature system of the tumor. It has been shown that p53 in combination with TSPf reduce tumors more effectively than p53 alone by gene therapy in mice tumor model. pRB, p21, p16 and other inhibitors of cell cycle-dependent kinases are also shown to inhibit endothelial cell proloiferation and thus block angiogenesis *in vivo*. Von Hippel Lindau gene (VHL) gene mutation is the most characterized in renal carcinoma. Mution of VHL leads to high expression of VEGF and highly angiogenic. Delivery of wild-type VHL gene would reduce expression of VEGF and block angiogenesis.

3. Integrin αvβ3 blockage:

Integrin αvβ3 are functionally associated on the surface of angiogenic blood vessels. The RGD-containing peptide ligand for integrin αvβ3 can home to tumors when injected intravenously into tumor-bearing mice. This ligand has been recently used to target chemotherapeutic drug to tumor vasculature for cancer treament in mice model (Arap, W *et al.*, *Science*, 279:377, 1998). Disruption of interaction between integrin αvβ3 and matrix metalloproteinase 2 (MMP-2) by a truncated MMP-2 (PEX) was shown to block angiogenesis

4. Angiogenic factor receptor blockage:

Receptors for VEGF (flt and flk) and for bFGF (bFGF receptors and bFGF binding proteins) regulate angiogenic signaling of growth factors. Neutrolizing Antibody against the angiogenic factors could block angiogenesis and tumor growth. Alternatively, soluble receptor can compete with wild type receptor in growth factor binding thus block angiogenesis.

5. Cytokines and chemokines:

Cytokine such as IL-12 and IFN-α and chemokine such as IP-10 have been shown to be potent inhibitors of angiogenesis in addition to their immunoregulatory effects.

6. Thrombosis factor (stimulator of blood coagulation:

Tissue factor (TF) is the major initiating receptor for the thrombogenic cascades. A truncated TF (tTF) has been targeted to tumor vasculature by a bispecific antibody and cause tumor infarction in mice (Huang, X. *et al.*, *Science*, 275:547, 1997)

D. Formulations

Delivery and expression of nucleic acids in many formulations is limited due to degradation of the nucleic acids by components of organisms, such as nucleases. Thus, protection of the nucleic acids when delivered *in vivo* can greatly enhance the resulting expression, thereby enhancing a desired pharmacological or therapeutic effect. It was found

that certain types of compounds which interact with a nucleic acid (e.g., DNA) in solution but do not condense the nucleic acid provide *in vivo* protection to the nucleic acid, and correspondingly enhance the expression of an encoded gene product.

The use of delivery systems designed to interact with plasmids and protect plasmids from rapid extracellular nuclease degradation has been described [Mumper, R.J., et al., 1996, *Pharm. Res.* 13:701-709; Mumper, R.J., et al., 1997. Submitted to *Gene Therapy*]. A characteristic of the PINC systems is that they are non-condensing systems that allow the plasmid to maintain flexibility and diffuse freely throughout the muscle while being protected from nuclease degradation. While the PINC systems are primarily discussed below, it will be understood that cationic lipid based systems and systems utilizing both PINCS and cationic lipids are also within the scope of the present invention.

A common structural component of the PINC systems is that they are amphiphilic molecules, having both a hydrophilic and a hydrophobic portion. The hydrophilic portion of the PINC is meant to interact with plasmids by hydrogen bonding (via hydrogen bond acceptor or donor groups), Van der Waals interactions, or/and by ionic interactions. For example, PVP and N-methyl-2-pyrrolidone (NM2P) are hydrogen bond acceptors while PVA and PG are hydrogen bond donors.

All four molecules have been reported to form complexes with various (poly)anionic molecules [Buhler V., BASF Aktiengescellschaft Feinchemie, Ludwigshafen, pp 39-42; Galaev Y, et al., *J. Chrom. A.* 684:45-54 (1994); Tarantino R, et al. *J. Pharm. Sci.* 83:1213-1216 (1994); Zia, H., et al., *Pharm. Res.* 8:502-504 (1991);]. The hydrophobic portion of the PINC systems is designed to result in a coating on the plasmid rendering its surface more hydrophobic. Kabanov et al. have described previously the use of cationic polyvinyl derivatives for plasmid condensation designed to increase plasmid hydrophobicity, protect plasmid from nuclease degradation,

and increase its affinity for biological membranes [Kabanov, A.V., and Kabanov, V.A., 1995, *Bioconj. Chem.* 6:7-20; Kabanov, A.V., et al., 1991, *Biopolymers* 31:1437-1443; Yaroslavov, A.A., et al., 1996, *FEBS Letters* 384:177-180].

Substantial protective effect is observed; up to at least a one log enhancement of gene expression in rat muscle over plasmid formulated in saline has been demonstrated with these exemplary PINC systems. The expression of reporter genes in muscle using plasmids complexed with the PINC systems was more reproducible than when the plasmid was formulated in saline. For example, the coefficient of variation for reporter gene expression in muscle using plasmid formulated in saline was 96 $\pm$ 35% (n = 20 studies; 8-12 muscles/study) whereas with coefficient of variation with plasmids complexed with PINC systems was 40 $\pm$ 19% (n = 30 studies; 8-12 muscles/study). The high coefficient of variation for reporter gene expression with plasmid formulated in saline has been described previously [Davis, H.L., et al., 1993, *Hum. Gene Ther.* 4:151-9]. In addition, in contrast with the results for DNA:saline, there was no significant difference in gene expression in muscle when plasmid with different topologies were complexed with polyvinyl pyrrolidone (PVP). This suggests that PVP is able to protect all forms of the plasmid from rapid nuclease degradation.

1. <u>Summary of interactions between a PINC polymer (PVP) and plasmid</u>

Using molecular modeling, it has been demonstrated that an exemplary PINC polymer, PVP, forms hydrogen bonds with the base pairs of a plasmid within its major groove and results in a hydrophobic surface on the plasmid due to the vinyl backbone of PVP. These interactions are supported by the modulation of plasmid zeta potential by PVP as well as by the inhibition of ethidium bromide intercalation into complexed plasmid. Apparent binding between PVP and plasmid has been correlated to pH and salt concentration and have demonstrated the effect of these parameters on β-gal expression after intramuscular

injection of plasmid/PVP complexes [Mumper, R.J., et al., 1997. Submitted to *Gene Therapy*]. A summary of the physico-chemical properties of plasmid/PVP complexes is listed in Table I below.

Table I: Summary of the Physico-Chemical Properties of Plasmid/PVP Complexes

Method	Result
Molecular modeling	Hydrogen bonding and hydrophobic plasmid surface observed
Fourier-transformed Infra-red	Hydrogen bonding demonstrated
DNase I challenge	Decreased rate of plasmid degradation in the presence of PVP
Microtitration Calorimetry	Positive heats of reaction indicative of an endothermic process
Potentiometric titration	One unit pH drop when plasmid and PVP are complexed
Dynamic Dialysis	Rate of diffusion of PVP reduced in the presence of plasmid
Zeta potential modulation	Surface charge of plasmid decreased by PVP
Ethidium bromide Intercalation	Ethidium bromide intercalation reduced by plasmid/PVP complexation
Osmotic pressure	Hyper-osmotic formulation (i.e., 340 mOsm/kg H2O)
Luminescence Spectroscopy	Plasmid/PVP binding decreased in salt and/or at pH 7

2. Histology of expression in muscle

Immunohistochemistry for β-gal using a slide scanning technology has revealed the uniform distribution of β-gal expression sites across the whole cross-sections of rat tibialis muscles. Very localized areas were stained positive for β-gal when CMV-β-gal plasmid was formulated in saline. β-gal positive cells were observed exclusively around the needle tract when plasmid was injected in saline. This is in agreement with previously published results [Wolff, J.A., et al., 1990, *Science* 247:1465-68; Davis, H.L., et al., 1993,

Hum. Gene Ther. 4:151-9; Davis, H.L., et al., 1993, *Hum. Gene Ther.* 4:733-40].

In comparison, immunoreactivity for β-gal was observed in a wide area of muscle tissue after intramuscular injection of CMV-β-gal plasmid/PVP complex (1:17 w/w) in 150 mM NaCl. It appeared that the majority of positive muscle fibers were located at the edge of muscle bundles. Thus, staining for β-gal in rat muscle demonstrated that, using a plasmid/PVP complex, the number of muscle fibers stained positive for β-gal was approximately 8-fold greater than found using a saline formulation. Positively stained muscle fibers were also observed over a much larger area in the muscle tissue using the plasmid/PVP complex providing evidence that the injected plasmid was widely dispersed after intramuscular injection.

The enhanced plasmid distribution and expression in rat skeletal muscle was a result of both protection from extracellular nuclease degradation due to complexation and hyper-osmotic effects of the plasmid/PVP complex. However, Dowty and Wolff et al. have demonstrated that osmolarity, up to twice physiologic osmolarity, did not significantly effect gene expression in muscle [Dowty, M.E., and Wolff, J.A. In: J.A. Wolff (Ed.), 1994, *Gene Therapeutics: Methods and Applications of Direct Gene Transfer.* Birkhauser, Boston, pp. 82-98]. This suggests that the enhanced expression of plasmid due to PVP complexation is most likely due to nuclease protection and less to osmotic effects. Further, the surface modification of plasmids by PVP (e.g., increased hydrophobicity and decreased negative surface charge) may also facilitate the uptake of plasmids by muscle cells.

3. <u>Structure-activity relationship of PINC polymers</u>

There is a linear relationship between the structure of a series of co-polymers of vinyl pyrrolidone and vinyl acetate and the levels of gene expression in rat muscle. The substitution of some vinyl pyrrolidone monomers with vinyl acetate monomers in PVP resulted in a co-polymer with reduced

ability to form hydrogen bonds with plasmids. The reduced interaction subsequently led to decreased levels of gene expression in rat muscle after intramuscular injection. The expression of β-gal decreased linearly (R = 0.97) as the extent of vinyl pyrrolidone monomer (VPM) content in the co-polymers decreased.

These data demonstrate that pH and viscosity are not the most important parameters effecting delivery of plasmid to muscle cells since these values were equivalent for all complexes. These data suggest that enhanced binding of the PINC polymers to plasmid results in increased protection and bioavailability of plasmid in muscle.

4. Additional PINC systems

The structure-activity relationship described above can be used to design novel co-polymers that will also have enhanced interaction with plasmids. It is expected that there is "an interactive window of opportunity" whereby enhanced binding affinity of the PINC systems will result in a further enhancement of gene expression after their intramuscular injection due to more extensive protection of plasmids from nuclease degradation. It is expected that there will be an optimal interaction beyond which either condensation of plasmids will occur or "triplex" type formation, either of which can result in decreased bioavailability in muscle and consequently reduced gene expression.

As indicated above, the PINC compounds are generally amphiphilic compounds having both a hydrophobic portion and a hydrophilic portion. In many cases the hydrophilic portion is provided by a polar group. It is recognized in the art that such polar groups can be provided by groups such as, but not limited to, pyrrolidone, alcohol, acetate, amine or heterocyclic groups such as those shown on pp. 2-73 and 2-74 of CRC Handbook of Chemistry and Physics (72nd Edition), David R. Lide, editor, including pyrroles, pyrazoles, imidazoles, triazoles, dithiols, oxazoles, (iso)thiazoles, oxadiazoles, oxatriazoles, diaoxazoles, oxathioles, pyrones, dioxins,

pyridines, pyridazines, pyrimidines, pyrazines, piperazines, (iso)oxazines, indoles, indazoles, carpazoles, and purines and derivatives of these groups, hereby incorporated by reference.

Compounds also contain hydrophobic groups which, in the case of a polymer, are typically contained in the backbone of the molecule, but which may also be part of a non-polymeric molecule. Examples of such hydrophobic backbone groups include, but are not limited to, vinyls, ethyls, acrylates, acrylamides, esters, celluloses, amides, hydrides, ethers, carbonates, phosphazenes, sulfones, propylenes, and derivatives of these groups. The polarity characteristics of various groups are quite well known to those skilled in the art as illustrated, for example, by discussions of polarity in any introductory organic chemistry textbook.

The ability of such molecules to interact with nucleic acids is also understood by those skilled in the art, and can be predicted by the use of computer programs which model such intermolecular interactions. Alternatively or in addition to such modeling, effective compounds can readily be identified using one or more of such tests as 1) determination of inhibition of the rate of nuclease digestion, 2) alteration of the zeta potential of the DNA, which indicates coating of DNA, 3) or inhibition of the ability of intercalating agents, such as ethidium bromide to intercalate with DNA.

5. Targeting Ligands

In addition to the nucleic acid/PINC complexes described above for delivery and expression of nucleic acid sequences, in particular embodiments it is also useful to provide a targeting ligand in order to preferentially obtain expression in particular tissues, cells, or cellular regions or compartments.

Such a targeted PINC complex includes a PINC system (monomeric or polymeric PINC compound) complexed to plasmid (or other nucleic acid molecule). The PINC system is covalently or non-covalently attached to (bound to) a targeting ligand (TL) which binds to receptors having an

affinity for the ligand. Such receptors may be on the surface or within compartments of a cell. Such targeting provides enhanced uptake or intracellular trafficking of the nucleic acid.

The targeting ligand may include, but is not limited to, galactosyl residues, fucosal residues, mannosyl residues, carnitine derivatives, monoclonal antibodies, polyclonal antibodies, peptide ligands, and DNA-binding proteins. Examples of cells which may usefully be targeted include, but are not limited to, antigen-presenting cells, hepatocytes, myocytes, epithelial cells, endothelial cells, and cancer cells.

Formation of such a targeted complex is illustrated by the following example of covalently attached targeting ligand, (TL) to PINC system:

TL-PINC + Plasmid ----------> TL-PINC:::::Plasmid

Formation of such a targeted complex is also illustrated by the following example of non-covalently attached targeting ligand (TL) to PINC system

TL::::::PINC + Plasmid --------> TL::::::PINC::::::Plasmid

or alternatively,

PINC + Plasmid ----------> PINC:::::::Plasmid + TL ------ ---> TL::::::PINC:::::::Plasmid

In these examples :::::::: is non-covalent interaction such as ionic, hydrogen-bonding, Van der Waals interaction, hydrophobic interaction, or combinations of such interactions.

A targeting method for cytotoxic agents is described in Subramanian et al., International Application No. PCT/US96/08852, International Publication No. WO 96/39124, hereby incorporated by reference in its entirety, including any drawings. This application describes the use of polymer affinity systems for targeting cytotoxic materials using a two-step targeting method involving zip polymers, i.e., pairs of interacting polymers. An antibody attached to one of the interacting polymers binds to a cellular target. That polymer then acts as a target for a second polymer attached to a

cytotoxic agent. As referenced in Subramanian et al., other two-step (or multi-step) systems for delivery of toxic agents are also described.

In another aspect, nucleic acid coding sequences can be delivered and expressed using a two-step targeting approach involving a non-natural target for a PINC system or PINC-targeting ligand complex. Thus, for example, a PINC-plasmid complex can target a binding pair member which is itself attached to a ligand which binds to a cellular target (e.g., a MAB). Binding pairs for certain of the compounds identified herein as PINC compounds as identified in Subramanian et al. Alternatively, the PINC can be complexed to a tareting ligand, such as an antibody. That antibody can be targeted to a non-natural target which binds to, for example, a second antibody.

III. Model Systems for Evaluation of Anti-Angiogenic Constructs and Formulations

In accord with the concept of using anti-angiogenic expressing plasmid constructs and formulations in anti-cancer treatment, murine model systems were utilized based on murine tumor cell lines. The line primarily used was S.C. VII/SF, which is a cell line derived from murine squamous cell carcinoma (S.C.).

Squamous cell carcinoma of the head and neck begins with the cells lining the oral and pharyngeal cavities. Clinical disease progresses via infiltration and spreads into the underlying tissues and lymphatics. The undifferentiated, *in vivo* passage tumor line S.C. VII/SF displays this typical growth pattern. In addition, its rapid growth rate provides a relatively short test period for individual experiments. Other murine tumor cell lines include another SCC line KLN-205, a keratinocyte line I-7, and a colon adenocarcinoma line MC-38.

An optimal model system preferably satisfies the criteria based on having tumor growth rate *in vivo* (*i.e.*, tumors are ready for treatment in 4-10 days post implant), invasiveness,

and local spread similar to those observed in clinical disease, and providing accessibility for experimental treatment. As indicated, the SCC VII/SF cell line was utilized as the primary model system cell line. This cell line typically grows rapidly, resulting in death of untreated syngeneic mice 14-17 days after tumor cell implantation.

This cell line can be utilized in a variety of ways to provide model system suitable for a variety of different tests. Four such possibilities are described below.

First, SCCVII cells can be utilized in cell culture to provide an *in vitro* evaluation of anti-angiogenic agent expression construct and formulation characteristics, such as expression levels and cellular toxicities.

Second, the cells can be implanted subcutaneously in mice. This system can be utilized in tests in which accessibility of the implant site is beneficial. As an example, the method was utilized in evaluations of expression efficiencies based on the expression of chloramphenicol acetyltransferase (CAT).

Third, the cells can be implanted transcutaneously into the fascia of digastric muscle.

Fourth, the cells can be implanted transcutaneously into digrastric/mylohyoid muscles. The important features of models 3 and 4 are shown in the table below.

TABLE II

Comparison of submandibular tumor models

Feature	Mouse Tumor Model 3	Mouse Tumor Model 4
Tumor implant procedure	2-4 x 10^5 cells transcutaneously into fascia of digastric muscle	5 x 10^5 transcutaneously into digastric/mylohyoid muscles
Tumor growth and invasiveness characteristics	Prominent submandibular bulge; invasion of digastric/mylohyoid muscles and lymphatics	More variable, invasion of digastric/mylohyoid muscles and lymphatics
Treatment procedure (primary treatment)	Transcutaneous, needle inserted and moved within tumor to produce a 4 quadrant distribution of gene medicine	Lower jaw skin flap raised to expose tumor, needle inserted and moved within tumor to produce a 4 quadrant distribution of gene medicine
Days treated (post-implant)	Day 5, day 10 (both transcutaneously)	Day 5 (tumor exposed), day 8 (transcutaneously)
Measurement procedure	External calipering 2-3 x per week until death	First caliper when tumor exposed for treatment, second caliper at sacrifice
Advantages	Non-surgical, closed model allows larger experiments and more frequent treatments; Sacrifice unnecessary to caliper (=more time points)	Surgical, open model allows direct treatment of exposed tumor; Local inflammation from surgery may additionally stimulate immune response; More like clinical situation for protocol development
Disadvantages	Transcutaneous treatment is potentially less accurate and intensive; less like expected clinical treatments than surgical approaches	Labor intensive; Smaller, fewer experiments possible; Tumors deeper and more difficult to treat transcutaneously (for secondary treatments); Fewer treatments and caliperings possible

The tumor size treated in the mouse models is generally 20-50 mm^3. A 50 mm^3 mouse tumor is approximately equivalent to 150 cc^3 human tumor having an average diameter of about 6.6 cm. This tumor size is approximately 10-fold larger than the size proposed to be treated in the phase I clinical trials. This indicates that the mouse models are strongly biased towards over estimating the expected tumor burden in human patients.

IV. Formulations for *In Vivo* Delivery

A. General

While expression systems such as those described above provide the potential for expression when delivered to an appropriate location, it is beneficial to provide the expression system construct(s) in a delivery system which can assist both the delivery and the cellular uptake of the construct. Thus, this invention also provides particular formulations which include one or more expression system constructs (e.g., DNA plasmids as described above), and a protective, interactive non-condensing compound.

An additional significant factor relating to the plasmid construct is the percentage of plasmids which are in a supercoiled (SC) form rather than the open circular (OC) form.

B. Delivery and Expression

A variety of delivery methods can be used with the constructs and formulations described above, in particular, delivery by injection to the site of a tumor can be used. The submandibular tumor models utilized injection into four quadrants of the tumor being treated.

C. Anti-Cancer Efficacy of Human Anti-Angiogenic Formulations

The effects of the administration of the anti-angiogenic formulations described above were evaluated using the S.C. VII mouse tumor models. Plasmid constructs as described above were incorporated in delivery formulations. The formulations were delivered by injection. The effects of the expression of

the human anti-angiogenic plasmids in tumor cells on the progress of the mouse tumors demonstrates that such anti-angiogenic expression is effective against such tumors.

D. Toxicity Evaluation of Exemplary Formulations

The exemplary formulations do not show high cellular toxicity at the concentrations tested, suggesting that the formulations do not significantly kill cells by direct toxic action *in vivo*.

V. Administration

Administration as used herein refers to the route of introduction of a plasmid or carrier of DNA into the body. In addition to the methods of delivery described above, the expression systems constructs and the delivery system formulations can be administered by a variety of different methods.

Administration can be directly to a target tissue or by targeted delivery to the target tissue after systemic administration. In particular, the present invention can be used for treating disease by administration of the expression system or formulation to the body in order to establishing controlled expression of any specific nucleic acid sequence within tissues at certain levels that are useful for gene therapy.

The preferred means for administration of vector (plasmid) and use of formulations for delivery are described above. The preferred embodiments are by direct injection using needle injection.

The route of administration of any selected vector construct will depend on the particular use for the expression vectors. In general, a specific formulation for each vector construct used will focus on vector uptake with regard to the particular targeted tissue, followed by demonstration of efficacy. Uptake studies will include uptake assays to evaluate cellular uptake of the vectors and expression of the DNA of choice. Such assays will also determine the

localization of the target DNA after uptake, and establishing the requirements for maintenance of steady-state concentrations of expressed protein. Efficacy and cytotoxicity can then be tested. Toxicity will not only include cell viability but also cell function.

Muscle cells have the unique ability to take up DNA from the extracellular space after simple injection of DNA particles as a solution, suspension, or colloid into the muscle. Expression of DNA by this method can be sustained for several months.

Delivery of formulated DNA vectors involves incorporating DNA into macromolecular complexes that undergo endocytosis by the target cell. Such complexes may include lipids, proteins, carbohydrates, synthetic organic compounds, or inorganic compounds. Preferably, the complex includes DNA, a cationic lipid, and a neutral lipid in particular proportions. The characteristics of the complex formed with the vector (size, charge, surface characteristics, composition) determines the bioavailability of the vector within the body. Other elements of the formulation function as ligand which interact with specific receptors on the surface or interior of the cell. Other elements of the formulation function to enhance entry into the cell, release from the endosome, and entry into the nucleus.

Delivery can also be through use of DNA transporters. DNA transporters refers to molecules which bind to DNA vectors and are capable of being taken up by epidermal cells. DNA transporters contain a molecular complex capable of non-covalently binding to DNA and efficiently transporting the DNA through the cell membrane. It is preferable that the transporter also transport the DNA through the nuclear membrane. See, *e.g.*, the following applications all of which (including drawings) are hereby incorporated by reference herein: (1) Woo et al., U.S. Serial No. 07/855,389, entitled "A DNA Transporter System and Method of Use", filed March 20, 1992, now abandoned; (2) Woo et al., PCT/US93/02725,

International Publ. WO93/18759, entitled "A DNA Transporter System and Method of Use", (designating the U.S. and other countries) filed March 19, 1993; (3) continuation-in-part application by Woo et al., entitled "Nucleic Acid Transporter Systems and Methods of Use", filed December 14, 1993, U.S. Serial No. 08/167,641; (4) Szoka et al., U.S. Serial No. 07/913,669, entitled "Self-Assembling Polynucleotide Delivery System", filed July 14, 1992 and (5) Szoka et al., PCT/US93/03406, International Publ. WO93/19768 entitled "Self-Assembling Polynucleotide Delivery System", (designating the U.S. and other countries) filed April 5, 1993.

A DNA transporter system can consist of particles containing several elements that are independently and non-covalently bound to DNA. Each element consists of a ligand which recognizes specific receptors or other functional groups such as a protein complexed with a cationic group that binds to DNA. Examples of cations which may be used are spermine, spermine derivatives, histone, cationic peptides and/or polylysine. One element is capable of binding both to the DNA vector and to a cell surface receptor on the target cell. Examples of such elements are organic compounds which interact with the asialoglycoprotein receptor, the folate receptor, the mannose-6-phosphate receptor, or the carnitine receptor. A second element is capable of binding both to the DNA vector and to a receptor on the nuclear membrane. The nuclear ligand is capable of recognizing and transporting a transporter system through a nuclear membrane. An example of such ligand is the nuclear targeting sequence from SV40 large T antigen or histone. A third element is capable of binding to both the DNA vector and to elements which induce episomal lysis. Examples include inactivated virus particles such as adenovirus, peptides related to influenza virus hemagglutinin, or the GALA peptide described in the Szoka patent cited above.

Transfer of genes directly into a tumor has been very effective. Experiments show that administration by direct injection of DNA into tumor cells results in expression of the

gene in the area of injection. The injected DNA preferably persists in an unintegrated extrachromosomal state. This means of transfer is a preferred embodiment.

Administration may also involve lipids as described in preferred embodiments above. The lipids may form liposomes which are hollow spherical vesicles composed of lipids arranged in unilamellar, bilamellar, or multilamellar fashion and an internal aqueous space for entrapping water soluble compounds, such as DNA, ranging in size from 0.05 to several microns in diameter. Lipids may be useful without forming liposomes. Specific examples include the use of cationic lipids and complexes containing DOPE which interact with DNA and with the membrane of the target cell to facilitate entry of DNA into the cell.

Gene delivery can also be performed by transplanting genetically engineered cells. For example, immature muscle cells called myoblasts may be used to carry genes into the muscle fibers. Myoblast genetically engineered to express recombinant human growth hormone can secrete the growth hormone into the animal's blood. Secretion of the incorporated gene can be sustained over periods up to 3 months.

Myoblasts eventually differentiate and fuse to existing muscle tissue. Because the cell is incorporated into an existing structure, it is not just tolerated but nurtured. Myoblasts can easily be obtained by taking muscle tissue from an individual who needs gene therapy and the genetically engineered cells can also be easily put back with out causing damage to the patient's muscle. Similarly, keratinocytes may be used to delivery genes to tissues. Large numbers of keratinocytes can be generated by cultivation of a small biopsy. The cultures can be prepared as stratified sheets and when grafted to humans, generate epidermis which continues to improve in histotypic quality over many years. The keratinocytes are genetically engineered while in culture by transfecting the keratinocytes with the appropriate vector. Although keratinocytes are separated from the circulation by

the basement membrane dividing the epidermis from the dermis, human keratinocytes secrete into circulation the protein produced.

The chosen method of delivery should result in expression of the gene product encoded within the nucleic acid cassette at levels which exert an appropriate biological effect. The rate of expression will depend upon the disease, the pharmacokinetics of the vector and gene product, and the route of administration, but should be in the range 0.001-100 mg/kg of body weight /day, and preferably 0.01-10 mg/kg of body weight/day. This level is readily determinable by standard methods. It could be more or less depending on the optimal dosing. The duration of treatment will extend through the course of the disease symptoms, possibly continuously. The number of doses will depend upon the disease, delivery vehicle, and efficacy data from clinical trials.

Examples

The present invention will be more fully described in conjunction with the following specific examples which are not to be construed in any way as limiting the scope of the invention.

Example 1:

Cloning EC-specific enhancer and promoters

Promoters: Two sets of reporter vectors - CMV enh^+/pro^- and CMV enh^-/pro^- with either CAT (pCT1132 and pCT1133) or LUC (pLC1137 and pLC1138 as reporter gene) were constructed. SacI site is an unique site in thses vectors. The promoter sequence will be amplified from human genomic DNA by PCR with two primers (5′ primer, 3′primer with SacI site) followed by cloning into TA vector. The construct with right orientation (3′ is away from the SacI site on TA vector so that SacI digest give PCR insert). The SacI fragment will be inserted into SacI site of a vector above.

Multimerized endothelin enhancer: The endothelin enhancer (ETe) was synthesized with overhangs to create Bgl II and Bam HI sites as shown below.

gatctGTACTTCATACTTTTCATTCCAATGGGGTGACTTTGCTTCTGGAG

aCATGAAGTATGAAAAGTAAGGTTACCCCACTGAAACGAAGACCTCctag

This DNA fragment was multimerized by ligation at high concentration and digested with Bgl II and Bam HI to eliminate inverted and everted repeats. DNA species containing four and seven tandem copies were gel-purified and inserted into plasmids containing various endothelial-specific or proliferation-specific promoters.

Materials and Methods

Construction of EC-specific promoter-driven reporter constructs: Plasmids containing endothelial-specific promoters were constructed as follows. The minimal promoter sequences of endothelin-1 (ET-1), KDR/flk-1, ICAM-2, β3, and αV, and cell cycle-dependent genes (cyclin A, E2F1, or cdc6) were directly amplified by PCR from human genomic DNA. The amplified promoter sequence was then subcloned into pCR 2.1 (Invitrogen). The promoter sequence was then subcloned as a SacI-SacI fragment into an expression plasmid pLC1136 which contains the luciferase reporter gene, a synthetic intron, and the human growth hormone 3′ untranslated region/poly (A) signal to create the promoter specific expression constructs. Plasmids were grown under kanamycin selection in *E. coli* host strains DH5α and purified using alkaline lysis and chromatographic methods. Purified plasmid utilized for injections had the following specifications: < 50 Eu/mg endotoxin.

Endothelial Cell Culture and transfection

Human umbilical aortic endothelial cells (HUVEC) were grown in six well plates in EBM-1 media (Clonetics, Inc.) supplemented with 5% fetal bovine serum with supplement of endothelial cell growth factors. HUVEC at passages 2-4 were used for transfection. HeLa cells were also grown in 6-well plates in Dulbecco's modified Eagle's medium supplemented with

10% fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin. Cultured cells were transfected by DEAE-dextran with 2 μg of reporter constructs. To correct for transfection efficiency, 0.5 μg of the plasmid, pBG0965, expressing the β-galactosidase gene, driven by the cytomeglovirus immediate early promoter, was also included in each transfection. Cell extracts were prepared 48 hr after transfection and luciferase and β-galactosidase assays were performed. The relative luciferase activity was calculated as the ratio of light units to β-galactosidase units. The corrected light units for HUVECs were divided by the corrected light units for HeLa cells to obtain fold endothelial-specificity.

Analysis of proliferating cell-specific promoters: The resulting reporter plasmids containing EC-enhancer and cell-cylce-specific promter into two different endothelial cell lines, HUVEC and BAEC, and compared the activity to that in a non-endothelial cell line, NIH3T3. The reporter activities was assayed in these samples.

Results

Construction of plasmids: The resulting reporter constructs are listed in the figures as are the representative constructs ET-enhancer/ET-promoter, pLC1264, and pLC1265.

EC-specific activity analysis: The activity of ETe/ETp in HUVEC and HeLa cells and the specificity (EC vs HeLa) are shown in the figures. The data indicated that ETe enhances ETp expression specifically in EC by 10-fold.

Four or seven copies of the ETe inserted upstream of the cyclin A, E2F1, or cdc6 promoter increases expression several fold specifically in endothelial cells. These chimeric regulatory elements, consisting of a proliferation-specific promoter and an endothelial-specific enhancer, may provide a means to achieve robust expression specifically in dividing endothelial cells *in vivo*.

EC-specific expression of anti-angiogenic genes: The coding sequence for endostatin or angiostatin was inserted into vector pLC1265 to generate pES1358 and pAS1359. Thus, the expression of endostatin and angiostatin was driven by ETe/ETp. The specificity of endostatin and angiostatin expression will be determinced.

Example 2: Anti-angiogenic gene medicines for cancer therapy

Materials and Methods

Plasmid construction: Plasmids containing an expression cassette for endostatin or angiostatin were constructed as follows. The coding sequences of endostatin is the 184 aa of C-terminal of collagen 18a1. (human collagen type XVIII alpha 1 Accession # L22548; Oh, S.P., Warman, M.L., Seldin, M. F., Cheng, S.D., Knoll, J. H., Timmons, S., and Olsen, B. R. Cloning of cDNA and genomic DNA encoding human type XVIII collagen and localization of the alpha 1(XVIII) colagen gene to mouse chromosome 10 and human chromosome 21. Genomics 19(3), 494-499 (1994). Angiostatin is internal fragment (97-440aa) of human plasminogen (Accession # M74220; Browne, M.J., Chapman, C.G., Dodd, I., Carey, J.E., Lawrence, G.M.P., Mitchell, D., and Robinson, J. H. Expression of recombinant human plasminogen and aglycolplasminiogen in HeLa cells. Fibrinolysis, (1991) Endostatin and angiostatin coding sequences were directly amplified by PCR from human liver cDNA (Clontech) using oligonucleotide primers (shown below), which add a BamHI site at the 5'end and an XbaI site at the 3'end:

human angiostatin 5' primer

ATg gAA CAT AAg gAA gTg gTT CTT

human angiostatin 3' primer (with XhoI site)

gC CTCgAg gCA TTT TTT CAg gTT gCA gTA CTC

human angiostatin 3' primer (internal primer) K3

gC ggATcc AAA gTg TAT CTC TCA gAg TgC AAg

mouse angiostatin 5' primer

ATg gAC CAT AAg gAA gTA ATC CTT

mouse angiostatin 3' primer (with XhoI site added)

gC CTCgAg gCA CCg CTT CAg gTT gCA gTA TTC
mouse angiostatin 3' primer (internal primer) K3?
gC ggATCC gTg TAT CTg TCA gAA TgT AAg ACC
mouse endostatin 5' primer (with BamHI added)
gCggATCC CAT ACT CAT CAg gAC TTT CAg CCA
mouse endostatin 3' primer (with Xho I added)
gCCTCgAg CTA TTT ggA gAA AgA ggT CAT gAA
human endostatin 5' primer
gAATTC CAC AgC CAC CgC gAC TTC CAg CCg
human endostatin 3' primer
CTCgAg CTA CTT ggA ggC AgT CAT gAA gCT

The amplified endostatin or angiostatin sequence was then subcloned into pCR 2.1 (Invitrogen). The coding sequence for endostatin or angiostatin was then subcloned as a BamH1-XbaI fragment into SfiI-XbaI sites of an intermediate vector, pHook1 (Invitrogen). Thus the coding sequence for an Igk sigal peptide and HA epitope from influenza virus was upstream of that of endostatin or angiostatin. The coding sequence for Igk-HA-endostatin or -angiostatin was then subcloned as an BamH1-XbaI fragment into the expression plasmid containing the cytomegalovirus immediate early promoter, a synthetic intron, and the bovine growth hormone 3′ untranslated region/poly (A) signal to create the endostatin or angiostatin expression systems, pES1100 (murine endostatin, mE), pES1281 (human endostatin, hE), pAS1095 (human angiostatin k1-k4, hAk4), or pAS1096 (human angiostatin k1-k3, hAk3). The HA-epitope was deleted from pES1100 by recombinant PCR to generate expression plasmid for HA-free mouse endostatin (pES1062, mE-HA-) **(Fig. 1)**. Plasmid pVC0612 (empty plasmid, EP) contains expression elements including the cytomegalovirus immediate early promoter and the 3' UTR/poly(A) signal from the bovine growth gene in the pVC0289 backbone described by Alila et al., *Human Gene Therapy*, 8:1785-1795 (1997). Plasmid pVC0612 was used as a control plasmid in all *in vivo* experiments. Plasmids for intra-tumoral injection were grown under kanamycin selection in E. coli host strains DH5α and purified using alkaline lysis

and chromatographic methods. Purified plasmid utilized for intra-tumoral injections had the following specifications: < 50 Eu/mg endotoxin; <1% protein; and < 20% chromosomal DNA.

Plasmid formulation:

DNA/PVP complex: Purified expression plasmid and control plasmids were formulated at a concentration of 3 mg DNA/ml in a PINC delivery system as described previously (Mumper et al., *Pharmaceutical Research*, Vol. 13, No. 5, (1996) and Mumper et al., *Journal of Controlled Release*, 52:191-203 (1998))

Preparation of liposomes and DNA/lipid complexes: Small unilamellar vesicles (SUVs), composed of the cationic lipid DOTMA (N-(1-(2-3-dioleyloxy)propyl)-n-n-n-trimethylammonium chloride):cholesterol at 4:1 mole ratio, were prepared by extrusion (400 nm). Positively charged plasmid/lipid complexes were prepared at a 1:3 -/+ charge ratio in 10% (w/v) lactose by mixing the plasmid with the liposomes under controlled conditions (Freimark et al., *The Journal of Immunology*, 160:4580-4586 (1998)). The mean diameter and zeta potential of the complexes were characterized using dynamic light scattering and Doppler electrophoretic light scattering. The complexation efficiency was determined by agarose gel electrophoresis.

Cell culture: Cos-1 cells were cultured in DMEM. Endothelial cells (HUVEC, HLMEC and BAEC) were from Clonetics) and cultured in specified medium according to the Manufacturer. TS/A is a tumor cell line established by Dr. P. Nanni, University of Bologna, Italy, from the first *in vivo* transplant of a moderately differentiated mammary adenocarcinoma that spontaneously arose in a BALB/c mouse (Nanni et al., *Clin. Exp. Metastasis*, 1:373-376 (1983)) A number of pre-immunization-challenge experiments suggested that TS/A does not elicit long-lasting anti-tumor immunity Renca, a spontaneously arising murine renal cell carcinoma, and CT-26, a colon adenocarcinoma, were also used, as was lewis lung carcinoma (LLC, metastatic variant from ATCC). Tumor cell cultures were maintained in sterile disposable

flasks from Corning (Corning, NY) at 37° C in a humidified 5% CO_2 atmosphere, using either RPMI 1640 (Renca, TS/A) or DMEM (LLC) supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin and 50 µg/ml gentamycin; all from Life Technologies.

Production of mouse antibody against human endostatin and angiostatin:

75 µµg of pES1281 or pAS1096 formulated in saline was injected intramuscularly and boosted on day 14 and 28. Antibodies were determined by western blotting.

Western blot analysis of endostatin and angiostatin: Cos cells, Ecs or tumor cells were plated in 6 well plates at 2.5 x 10^5 cells/well, and transfected using 2 µg of plasmids pES1100, pES1062, pES1281, pAS1095, or pAS1096 and 3 µg of Lipofectamine (Life Technologies, Inc., Gaithersburg, MD) in serum-free DMEM. Supernatants were harvested 24 hours later and endostatin or angiostatin were immunoprecipitated using mouse monoclonal anti-HA and protein A and G agarose (Boehringer Mannheim, Indianapolis, IN). Samples were run on a 12% Tris-glycine gel and electroblotted to Millipore PVDF membrane. Monoclonal anti-HA (Boehringer Mannheim), anti-angiostatin (IgG (Enzyme Research Laboratories, Inc.) or anti-endostatin was used at 1:1000, followed by anti-mouse (or rabbit) Ig HRP (Amersham Life Science) at 1:1000. Rainbow molecular weight markers (Amersham Life Science) were used to determine protein size. Detection was performed using the Amersham ECL kit.

Angiostatin/Endostatin ELISA: Supernatants (1 ml/well) were collected from transfected cells above and levels of endostatin or angiostatin were assayed by enzyme-linked immunosorbent assay (ELISA). ELISA plates (Falcon flexible PVC #3912) were coated with affinity purified goat anti-PG (plasminogen) IgG (Enzyme Research Laboratories, Inc.) or anti-endostatin (Folkman lab) in 50 mM carbonate buffer (1/500 dilution) at 4° C overnight and blocked with 150 µl/well of 2% BSA in PBS at room temperature for 4 hr. Supernatants (100

µl/well) were added and incubated for 1 hr at 37° C. Mouse monoclonal anti-HA (Boehringer Mannheim) diluted 1/500 in HBS-Tween-BSA buffer (23.8 M HEPES, 5.84 M NaCl, 1.0% BSA, 0.1% Tween; pH 7.2) was added (100 µl/well) and incubated for 1 hr at 37 ° C. Peroxidase conjugated anti-mouse IgG (Amersham Life Sciences) diluted 1/500 in HBS-Tween-BSA buffer was added (100 µl/well) and incubated for 1 hr at 37° C. 100µl/well of OPD substrate (o-phenylenediamine (Sigma 5 mg tablets) diluted in citrate-phosphate buffer (5.2 M citric acid, 13.8 M Na_2HPO_4; pH 5) with 0.1% H_20_2 was applied and color was developed for 2-5 min. The reaction was stopped by the addition of 50µl/well of 2.5 M H_2SO_4. Absorbance was determined at 450 nm in an EL340 Microplate reader (Bio-tek Instruments). Serial two-fold dilutions of plasminogen lysine-binding site I (Sigma), HA-angiostatin or HA-endostatin from transfected cells was used as a standard.

Animals: Normal 8-week-old female BALB/c or C57bl mice were purchased from Harlan Laboratories, Houston, TX. Mice were maintained on ad libitum rodent feed and water at 23^0 C, 40% humidity, and a 12-h/12-h light-dark cycle. Animals were acclimated for at least 4 days before the start of the study.

In vivo evaluation of tumor growth and treatments: BALB/c or C57bl mice were challenged s.c. in the middle of the left flank with 30 µµl of a single-cell suspension contained the specified number of cells. Seven days later when the tumor size reached approximately 10 mm^3, treatments with endostatin/PVP or EP/PVP started and were repeated at 1-2 day intervals for 2 weeks (total of 8 treatments: 4/week). Tumor volume was measured with electronic caliper in the two perpendicular diameters and in the depth. Measurements of the tumor masses (mm^3) were performed twice a week for 40-50 days. All mice bearing tumor masses exceeding 1 cm^3 volume were sacrificed for humane reasons. Data for the effects of endostatin or angiostatin gene therapy on tumor growth were analyzed by repeated measures analysis. Individual treatment means were compared using Duncan's multiple range test when

the main effect was significant. Data for the effect of endostatin or angiostatin gene therapy on tumor rejection were analyzed by ANOVA. In all cases a p value of less than 0.05 was considered to be statistically significant.

In vivo evaluation of lung metastases and treatments:

Lung metastases were established in mice by injecting 3 x 10^5 Renca cells in 100 µL HBSS (Hank's Balanced Salt Solution, without Ca^{++} or Mg^{++}, Life Technologies) into the tail vein. Animals were warmed using a 150-watt lamp and placed in a mouse restrainer prior to tail vein injection. 4 or 7 day after tumor injection, mice were injected intravenously with endostatin/DC, angiostatin/DC or EP/Dconce a week for 2-3 weeks. The lungs were insufflated with 1-2 mL India ink solution (150 mL distilled H_2O, 30 mL India ink, 4 drops ammonium hydroxide), using a 22 gauge gavage needle, then fixed with Fekete's solution (90 mL Formaldehyde, 37% solution, 900 mL 70% EtOH, 45 mL glacial acetic acid) for at least 24 hours. Metastases counts were performed under a dissecting microscope. Survival data were analyzed using the Kaplan-Meier log-ranked test. All other data were analyzed using the Neuman-Keul's Test on StatMost for Windows software (DataMost Corporation, Sandy, UT). Data were considered statistically significant if p values were < 0.05.

LLC model: Subcutaneous tumor (8-12 mm in diameter) were resected aseptically. All necrotic zones were removed and the viable tissue was minced and dissociated with collagen (Type I, 200 U/ml) and Dnase (270 U/ml) (Sigma Chemical Co., St. Louis, MO). Cells were susupended in DMED with nsupplement and plated at 5-10x106 viable cells/T175 flask. After a 3 hr adherence, the culture were rinsed and given fresh medium. 48 h later, te adherent tumor cells were harvested by brief trypsinization, wahsed once with medium and resuspend in HBSS. Aliquotes of 106 cells in 0.1 ml of HBSS were injected subcutaneously. When tumors were 12-15 in diameter, the mice were anesthetized with methoxyflurane. The tumors in one group of mice were surgical excised, and the area closed with

metal woud clips. The other group of mice underwent a sham surgical procedure which left the sc tumors intact. The mice were monitored daily and killed 10-14 days after surgery. The lungs were weighed and stained as described above.

Intramuscular injection and electroporation: 200-300 ug of DNA/PVP (3mg/ml) were injected into tibialis (25 ul) and gastrocnemia (50 ul) in one mouse leg, 2 min after injection, electroporation (500V/cm, 96 usec with 4 pulses) was applied on the injected leg.

Histological analysis: For CD-31, CD3, CD4, MAC-1 Immunostaining: Frozen sections were cut at 5μM and then subsequently fixed in Acetone for 10 minutes at room temperature. Immunohistochemistry was accomplished utilizing an avidin biotin technique. Endogenous peroxidases were quenched by incubating the sections with a 1% H_2O_2 solution for 10 minutes at room temperature. Nonspecific binding was blocked with an incubation of 5 minutes at room temperature with PowerBlock (Cat# HK085-5K, Biogenex, San Ramon, CA, USA). The sections were then incubated with the appropriate dilution of primary antibody for 1 hour at room temperature. Following washes in PBS, the secondary antibody, biotinylated anti-Rat (Cat#BA-4001, Vector Laboratories, Burlingame, CA, USA) 1:400, was added and the sections were incubated for 1 hour at room temperature. Following washes in PBS, the vectastain reagent (Cat#pk-6001, Vector Laboratories, Burlingame, CA, USA) was added at a dilution of 1:80 and the sections were incubated for 1 hour at room temperature. 3,3-'diaminobenzidine (Stable DAB, cat#750118, Research Genetics, Huntsville, AL, USA) was used as the chromagen. The primary antibodies used were all Rat anti-Mouse Monoclonal Antibodies. The antibodies and dilutions were: CD3 (Cat#19914-D19. Gibco, Gaithersburg, MD, USA) 1:250, CD4 (Cat#380220, Seikagaku, America, Inc., Ijamsville, MD, USA) 1:10,000, MAC-1 (Cat# CL8941AP, Cedar Lane Laboratories, LTD, Westbury, NY, USA) 1:100, CD31 (Cat#0951D, Pharmingen, San Diego, CA, USA) 1:800.

Apoptotic cells were detected utilizing the ApopTag inSitu Apoptosis detection kit (Cat# S7100-KIT, Oncor, Gathersburg, MD, USA). The residues of digoxigenin-nucleotide were catalitically added to the DNA by terminal deoxynucleotidyl transferase.

PCNA immunostaining was accomplished utilizing the Mouse to Mouse HRP kit (Cat#MTM001, Scytek, Logan, UT, USA) and following the manufacturer's instructions. The PCNA antibody (Cat#32251, Pharmingen, San Diego, CA, USA) at a dilution of 1:10,000.

Anti-angiogenesis assay: *In vitro* inhibition of EC proliferation assay was performed as follows. 5,000 cells of human lung microvessel nendothelial cells (HLMEC) were plated onto gelatinized 96-well culture plates and incubate (37°C, 5% CO2) for 24 h in 100ul HLMEC medium containing growth factors (bFGF). The media was replaced with 80ul of endostatin- or angiostatin-containing supernatant from transfected cells. After 20 min of incubation, 20 ul of HLMEC media was added. After 72 h, cell numbers were analyzed by WST-1 assay (Boeringer).

In vivo neovascularization was assayed in mouse cornea. Hydron pellet (Hydron; Interferon Sciences) of <1 ul (Aluminum sucrose sulfate; Sigma) were formulated containing bFGF at 1 ug/ml and implanted into the cornea of C57bl mouse 0.3-0.5 mm from the limbus. Next day, DNA/PVP was injected im or DNA/DC was injected iv. Angiogenesis was assessed by slit-lamp microscopy on day 3, 4, and 5 after implantation. Maximal angiogenesis was achieved on day 5. Inhibition of angiogenesis by endostatin or angiostatin gene medicine was examined by comparing to control vector.

Results

Construction of expression plasmids for endostatin and angiostatin: The coding sequence for endostatin or angiostatin (k1-k3) was PCR amplified from liver cDNA library since collagen 18a (endostatin precusor) and plasminogen (angiostatin precusors are rich in liver). Due to the fact

that Angiostatin and Endostatin are internal fragment devoid of their natural secretion sequence, and that there are no commercially available antibodies against angiostatin or endostatin, HA epitope which is 9aa from influenza hemaglutinin protein is tagged to the N-terminus of angiostatin or endostatin so that antibody against this peptide can be used for detection. Ig-kappa signal peptide is added to upstream of HA-angiostatin or -endostatin to direct secretion of the fusion protein. The expression plasmids for HA-tagged mouse endostatin (mE, pES1100), HA-free mouse endostatin (mE-HA$^-$, pES1062), HA-human endostatin (hE, pES1281), HA-human angiostatin k1-k4 (hAk4), HA-angiostatin k1-k3 (hAk3, pAS1096) were constructed and the maps were shown in **Fig.1**.

Expression of bioactive endostatin and angiostatin: To assess their expression, the expression plasmids are transfected into cos-1 cells, human endothelial cells (HUVEC), or Renca tumor cells. The transgene expression was examined for mRNA in cells by RT-PCR and for protein in media by western blotting (**Fig 2**). The results showed Endostatin and Angiostatin were transcribed as correct sizes as indicated and no missplicing products were detected. The recombinant protein was present as a single band with an approximate molecular weight of 22 KD (HA-mE), 21 KD (mE), 20 KD (HA-hE) or 30 KD (HA-hAk3). The protein expression was quantitatively assessed an ELISA with anti-Endosattin or plasminogen antibody and anti-HA epitope antibody. The result indicated that mE and hAk3 was about 4 ng/ml in the culture medium. hAk3 was expressed at higher level than hAk4. mE-HA$^-$ expression was determined by western blttoing with anti-endosattin antibody. The conditioned media containing endostatin and angiostatin showed strong inhibitory effects on endothelial cell proliferation.

In vivo expression

Intratumoral injection: 24 ug DNA/PVP was injected intratumorally and tumor was harvested at 24 h. The protein

expression of endostatin or angiostatin was determined by ELISA. The tumor culture media was also examined for bioactivity of endostatin and angiostatin.

Intramuscular delivery: 400-420 μg DNA/PVP was injected intramulscularly followed by electroporation (see Materials and Methods). Serum was collected at day 2, 5 and 10. The data showed that 3-5 ng/ml of Endostatin or 10-15 ng/ml of Angiostatin in serum was produced on day 5, and electroporation increased the level of trangenes by 3-5-fold. The expression declined on day 10.

Intravascular delivery: DOTMA:chol formulated endostatin or angiostatin expression plasmid was injected i.v. to normal mice. Expression of endostatin and angiostatin were determined by RT-PCR for mRNA in lung and ELISA for protein expression in serum. 400 nm size particle resulted in higher expression and may have more secondary cytokine effect than the SUV formulation (see *in vivo* efficacy).

In vivo efficacy: Three approaches to assess Endostatin *in vivo* anti-tumor activity are described below- sc tumor by intratumoral injection, sc tumor by im injection and lung tumor model by systemic delivery (iv and im). The data from these three models is summarized below.

Renca s.c. tumor model with intratumoral injection: Intratumoral administration of 24 μg Endostatin gene/PVP at 4 times/week for two weeks (total 7 treatments) induced complete regression in 7 out of 14 mice (50% regression rate, $p<0.05$). Survival rate was increased from 21% (3/14) in vector/PVP group to 78% (11/14) in Endostatin/PVP group on day 21. Human endostatin with HA-tag was constructed. Intratumoral injection of human endostatin/PVP resulted in 51% tumor growth inhibition in Renca sc tumor model.

The regressed tumors by Endostatin gene medicine remain in a microscopic dormant state by day 29, whereas 6 cycles treatments (400 μg/mouse/day for 27 days each cycle) with recombinant protein is needed to keep tumor dormancy (Boehm et al., *Nature* **390**, 404-7). The data presented herein clearly

demonstrates that Endostatin gene medicine has strong anti-tumor activity in mouse tumor model, and may have many advantages over the recombinant protein. Histological analysis of the regressing tumors by Endostatin/PVP treatment showed 3-5-fold reduced vascularization (CD31 staining), 3-fold increased tumor apoptosis (TdT immunostaining). No change in tumor proliferation (PCNA staining) and tumor infiltration lymphocytes (CD3, CD4 and Mac-1 staining).

Renca s.c. tumor with systemic delivery: Im injection of 120 μg of Endostatin/PVP followed by electroporation (2x week, switch legs) induced 40% Renca tumor injection. The regressing tumors had reduced vascularization. Repeated experiment showed that 34 % tumor growth inhibition and prolonged survival.

However, intravascular delivery of vector or Endostatin/Dotma:chol (SUV, 30 μg/2x week) didn't show a gene-specific effect, although overall 50-60% reduction of tumor growth was observed. Use of other lipids is expected to yield improved results.

Lung metastasis model with systemic delivery: It has been shown that IL-12 at 15 μg DNA in 400 nm particle induced prolonged survival. It is ongoing to see if Endostatin, Angiostatin can prolong survival by iv or im delivery of the gene medicine.

LLC can metastize to lung when sc primary tumor is excised. This lung metastasis model will be established to assess inhibitory effects of lung metastases by anti-angiogenic gene medicines.

Mouse cornea assay: Mouse cornea angiogenesis was induced by implantating bFGF pellet. Endostatin gene medicine was delivered by either iv of DNA/DC or DNA/PVP on next day (see Materials and Methods). The results showed Endostatin by iv strongly inhibited bFGF-induced cornea angiogenesis by day 5.

New anti-angiogenic genes: A double-barrel expression plasmid for endostatin-angiostatin (pAS1254), in which

endostatin and angiostatin are driven by separate transcription units, has also been also constructed.

IP-10, interferon-inducing factor 10, is a potent chemokine and anti-angiogenic factor. Thrombospondin-1 (TSP-1), a p53-induced glycoprotein on EC surface, is a potent inhibitor of angiogensis. An internal fragment (TSPf) is essential for its activity. IP-10 cDNA was reverse transcribed from published protein sequence with optimal humanized codons. The cDNA was synthesized by Operon and the expression plasmids for IP-10 (pIP-1316), IP-10/endostatin fusion protein (pIP1311), and IP-10/TSPf fusion protein have been constructed. The expression of IP-10, IP-10/hE fusion protein, and IP-10/TSPf fusion protein were determined by RT-PCR for mRNA and by western blotting for protein. About 150-250 ng/ml protein was expressed in culture medium. These constructs will be exmained in Renca sc model to see if synergistic anti-tumor efficacy could be achieved.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same

extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. For example, if X is described as selected from the group consisting of bromine, chlorine, and iodine, claims for X being bromine and claims for X being bromine and chlorine are fully described.

Other embodiments are within the following claims.

Claims

1. A plasmid comprising a tissue specific element transcriptionally linked an anti-angiogenic coding sequence.

2. The plasmid of claim 1, wherein said tissue specific element is specific for endothelial cells.

3. The plasmid of claim 1, wherein said tissue specific element comprises a promoter.

4. The plasmid of claim 3, wherein said promoter is selected from the group consisting of ET-1, flk-1, Alpha-V, Beta-3, ICAM-2, cyc A, E2F1, and cdc6.

5. The plasmid of claim 1, wherein said tissue specific element comprises an enhancer.

6. The plasmid of claim 5, wherein said enhancer is selected from the group consisting of CMV, four copies of ET-1, and seven copies of ET-1.

7. The plasmid of claim 1, wherein said coding sequence encodes a product selected from the group consisting of endostatin, angiostatin, thrombspondin-1, p53, IL-12, IFN-alpha, truncated tissue factor, an integrin $\alpha v\beta 3$ blocking agent, a VHL gene product, a cell cycle-dependant kinase inhibitor, a VEGFr, bFGFr, and a bFGF binding protein.

8. The plasmid of claim 7, wherein coding sequence is a synthetic sequence having optimal codon usage.

9. The plasmid of claim 1, wherein said coding sequence has the nucleotide sequence of plasmid pES1281, pIP1316, pAS1095 or pAS 1096.

10. The plasmid of claim 1, further comprising a growth hormone 3' untranslated region.

11. The plasmid of claim 10, wherein said growth hormone 3' untranslated region is from a human growth hormone gene.

12. The plasmid of claim 10, wherein an ALU repeat or ALU repeat-like sequence is deleted from said 3' untranslated region.

13. The plasmid of claim 1, wherein said plasmid includes a promoter, a TATA box, a Cap site and a first intron and intron/exon boundary in appropriate relationship for expression of said coding sequence.

14. The plasmid of claim 13, wherein said plasmid further comprises a 5' mRNA leader sequence inserted between said promoter and said coding sequence.

15. The plasmid of claim 1, wherein said plasmid further comprises an intron/5' UTR from a chicken skeletal α-actin gene.

16. The plasmid of claim 1, further comprising:

a first transcription unit comprising a first transcriptional control sequence transcriptionally linked with a first 5'-untranslated region, a first intron, a first coding sequence, and a first 3'-untranslated region/poly(A) signal, wherein said first intron is between said control sequence and said first coding sequence; and

a second transcription unit comprising a second transcriptional control sequence transcriptionally linked with a second 5'-untranslated region, a second intron, a second coding sequence, and a second 3'-untranslated region/poly(A) signal, wherein said second intron is between said control sequence and said second coding sequence;

wherein said first and second coding sequences comprise a sequence coding for angiostatin, and a sequence coding for endostatin.

17. The plasmid of claim 16, wherein said first transcriptional control sequence or said second transcriptional control sequence comprise one or more cytomegalovirus promoter sequences.

18. The plasmid of claim 16, wherein said first and second transcriptional control sequences are the same.

19. The plasmid of claim 16, wherein said first and second transcriptional control sequences are different.

20. The plasmid of claim 19, wherein said sequence coding for angiostatin is 5' to said sequence coding for endostatin.

21. The plasmid of claim 1, further comprising an intron having variable splicing, a first coding sequence, and a second coding sequence,

wherein said first and second coding sequences comprise a sequence coding for angiostatin, and a sequence having the sequence coding for endostatin.

22. The plasmid of claim 21, further comprising:

a transcriptional control sequence transcriptionally linked with a first coding sequence and a second coding sequence;

a 5'-untranslated region;

an intron 5' to said first coding sequence;

an alternative splice site 3' to said first coding sequence and 5' to said second coding sequence; and

a 3'-untranslated region/poly(A) signal.

23. The plasmid of claim 22, wherein said transcriptional control sequence comprises a cytomegalovirus promoter sequence.

24. The plasmid of claim 1, further comprising:

a transcriptional control sequence transcriptionally linked with a first coding sequence, an IRES sequence, a second coding sequence, and a 3'-untranslated region/poly(A) signal, wherein said IRES sequence is between said first coding sequence and said second coding sequence; and

an intron between said promoter and said first coding sequence;

wherein said first and second coding sequences comprise a sequence coding for angiostatin, and a sequence coding endostatin.

25. The plasmid of claim 24, wherein said transcriptional control sequence comprises a cytomegalovirus promoter sequence.

26. The plasmid of claim 24, wherein said IRES sequence is from an encephalomyocarditis virus.

27. A composition comprising the plasmid of anyone of claims 1-26, and a protective, interactive non-condensing compound.

28. The composition of claim 27, wherein said protective, interactive non-condensing compound is polyvinyl pyrrolidone.

29. The composition of claim 27, wherein said plasmid is in a solution having between 0.5% and 50% PVP.

30. The composition of claim 29, wherein said solution includes about 5% PVP.

31. The composition of claim 27, wherein said DNA is at least about 80% supercoiled.

32. The composition of claim 31, wherein said DNA is at least about 90% supercoiled.

33. The composition of claim 32, wherein said DNA is at least about 95% supercoiled.

34. A composition comprising a protective, interactive non-condensing compound and a plasmid comprising an anti-angiogenic coding sequence.

35. A composition comprising the plasmid of any one of claims 1-26 and a cationic lipid.

36. The composition of claim 35, further comprising a neutral co-lipid.

37. The composition of claim 35, wherein said cationic lipid is DOTMA.

38. The composition of claim 36, wherein said neutral co-lipid is cholesterol.

39. The composition of claim 35, wherein the DNA in said plasmid and said cationic lipid are present in such amounts that the negative to positive charge ratio is between 1:0.1 and 1:10.

40. The composition of claim 39, wherein said ration is between 1:0.3 and 1:6.

41. The composition of claim 40, wherein said ratio is about 1:3.

42. The composition of claim 35, wherein said DNA is at least about 80% supercoiled.

43. The composition of claim 42, wherein said DNA is at least about 90% supercoiled.

44. The composition of claim 43, wherein said DNA is at least about 95% supercoiled.

45. The composition of claim 35, further comprising an isotonic carbohydrate solution.

46. The composition of claim 45, wherein said isotonic carbohydrate solution consists essentially of about 10% lactose.

47. The composition of claim 36, wherein said cationic lipid and said neutral co-lipid are prepared as a liposome having an extrusion size of between 100 and 1,000 nanometers.

48. The composition of claim 47, wherein said size is between 200 and 900 nanometers.

49. The composition of claim 48, wherein said size is about 400 nanometers.

50. A composition comprising:

a first component comprising a plasmid comprising an anti-angiogenic coding sequence and a cationic lipid with a neutral co-lipid, wherein said cationic lipid is DOTMA and said neutral co-lipid is cholesterol, wherein the DNA in said plasmid and said cationic lipid are present in amounts such that the negative to positive charge ratio is about 1:3; and

a second component comprising a protective, interactive non-condensing compound, wherein said first component is present within the second component.

51. A composition comprising a protective, interactive non-condensing compound, a first plasmid comprising an angiostatin coding sequence, and one or more other plasmids independently comprising an endostatin coding sequence.

52. A composition comprising a plasmid comprising an anti-angiogenic coding sequence and a cationic lipid with a neutral co-lipid.

53. A method for making a plasmid of anyone of claims 1-26 comprising the step of inserting an anti-angiogenic coding sequence and a tissue specific element into a plasmid.

54. The method of claim 53, wherein said method further comprises transcriptionally linking said anti-angiogenic coding sequence and said tissue specific element.

55. A method for making a composition of claim 34, comprising the steps of:

a. preparing a DNA molecule comprising a transcriptional unit, wherein said transcriptional unit comprises an anti-angiogenic coding sequence;

b. preparing a protective, interactive non-condensing compound; and

c. combining said protective, interactive non-condensing compound with said DNA in conditions such that a composition capable of delivering a therapeutically effective amount of an anti-angiogenic coding sequence to a mammal is formed.

56. The method of claim 55 wherein said DNA molecule is a plasmid, wherein said plasmid comprises an anti-angiogenic coding sequence, and a human growth hormone 3'-untranslated region/poly(A) signal.

57. A method of making a composition of claim 36, comprising the steps of:

a. preparing a DNA comprising an anti-angiogenic coding sequence;

b. preparing a mixture of a cationic lipid and a neutral co-lipid, wherein said cationic lipid is DOTMA and said neutral co-lipid is cholesterol; and

c. combining said mixture with said DNA in amounts such that said cationic lipid and said DNA are present in a negative to positive charge ratio of about 1:3.

58. A method of making a composition of claim 50, comprising the steps of:

a. preparing a first component comprising a plasmid comprising an anti-angiogenic coding sequence and a cationic lipid with a neutral co-lipid, wherein said cationic lipid is DOTMA and said neutral co-lipid is cholesterol, wherein the DNA in said plasmid and said cationic lipid are present in amounts such that the negative to positive charge ratio is about 1:3;

b. preparing a second component comprising a protective, interactive non-condensing compound; and

c. combining said first and second components such that the resulting composition comprises said first component within said second component.

59. A method of making a composition of claim 51, comprising the steps of:

a. preparing a protective, interactive non-condensing compound,

b. preparing a first plasmid comprising an angiostatin coding sequence,

c. preparing one or more other plasmids independently comprising an endostatin coding sequence, and

d. combining said protective, interactive non-condensing compound, said plasmid comprising said angiostatin coding sequence and said other plasmids.

60. A method of making a composition of claim 52 comprising combing a plasmid comprising an anti-angiogenic coding sequence and a cationic lipid with a neutral co-lipid.

61. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a plasmid of anyone of claims 1-26.

62. The method of claim 61, wherein said method further comprises transiently expressing said plasmid.

63. The method of claim 61, wherein said condition or disease is a cancer.

64. The method of claim 63, wherein said composition is administered by injection.

65. A method for transfection of a cell *in situ*, comprising the step of contacting said cell with a plasmid of anyone of claims 1-26 for sufficient time to transfect said cell.

66. The method of claim 65, wherein transfection of said cell is performed *in vivo*.

67. The method of claim 65, wherein said contacting is performed in the presence of an about 5% PVP solution.

68. A method for delivery and expression of an anti-angiogenic gene in a plurality of cells, comprising the steps of:

(a) transfecting said plurality of cells with a plasmid of anyone of claims 1-26; and

(b) incubating said plurality of cells under conditions allowing expression of a nucleic acid sequence in said vector, wherein said nucleic acid sequence encodes an anti-angiogenic agent.

69. The method of claim 68, wherein said anti-angiogenic agent is angiostatin or endostatin and said cells are human cells.

70. The method of claim 68, wherein said contacting is performed in the presence of an about 5% PVP solution.

71. A method for treating a disease or condition, comprising the steps of transfecting a cell *in situ* with a plasmid of any one of claims 1-26.

72. The method of claim 71, wherein said disease or condition is a localized disease or condition.

73. The method of claim 72, wherein said disease or condition is a solid tumour.

74. The method of claim 71, wherein said disease of condition is a systemic disease or condition.

75. The method of claim 74, wherein said disease or condition is a metastatic cancer.

76. A cell transfected with a plasmid of anyone of claims 1-26.

77. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 27.

78. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 34.

79. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 35.

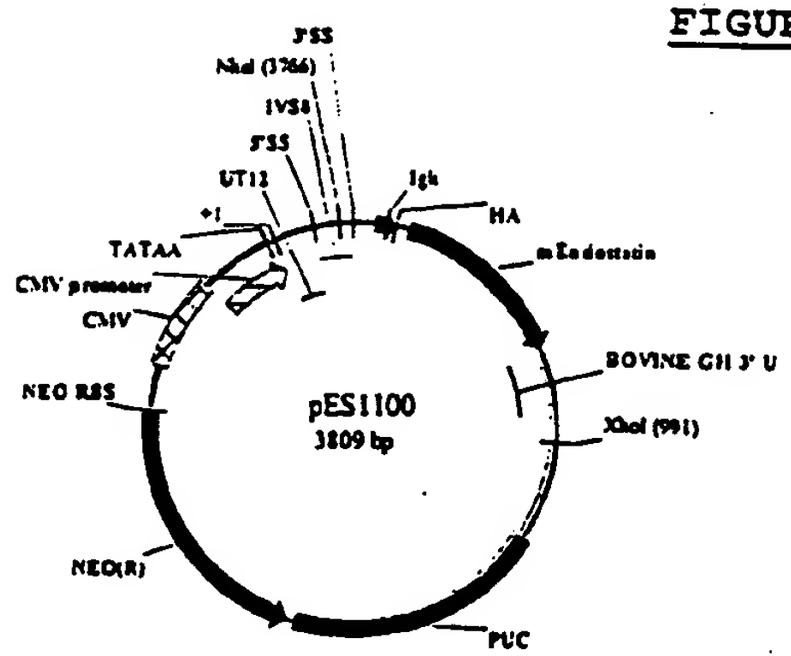
80. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 50.

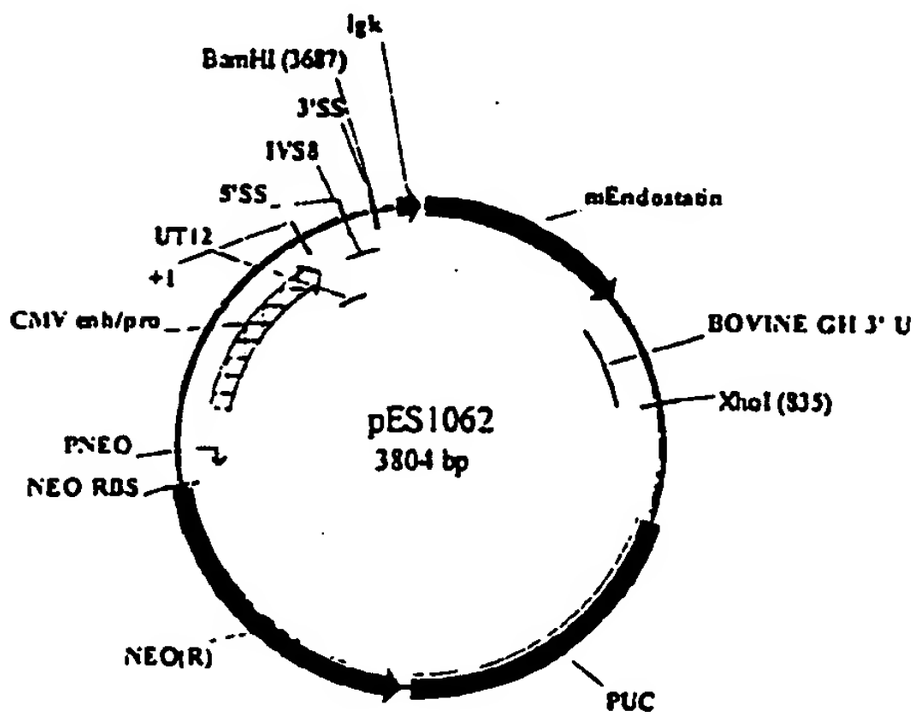
81. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 51.

82. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 52.

83. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of a first plasmid comprising an angiostatin coding sequence and a second plasmid comprising an endostatin coding sequence.

FIGURE 1


3'SS
NheI (3766)
IVS8
5'SS
UT12
+1
TATAA
CMV promoter
CMV
NEO RBS
NEO(R)
Igk
HA
mEndostatin
BOVINE GH 3' U
XhoI (991)
pES1100
3809 bp
PUC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BamHI (3687)
3'SS
IVS8
5'SS
UT12
+1
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NEO(R)
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XhoI (835)
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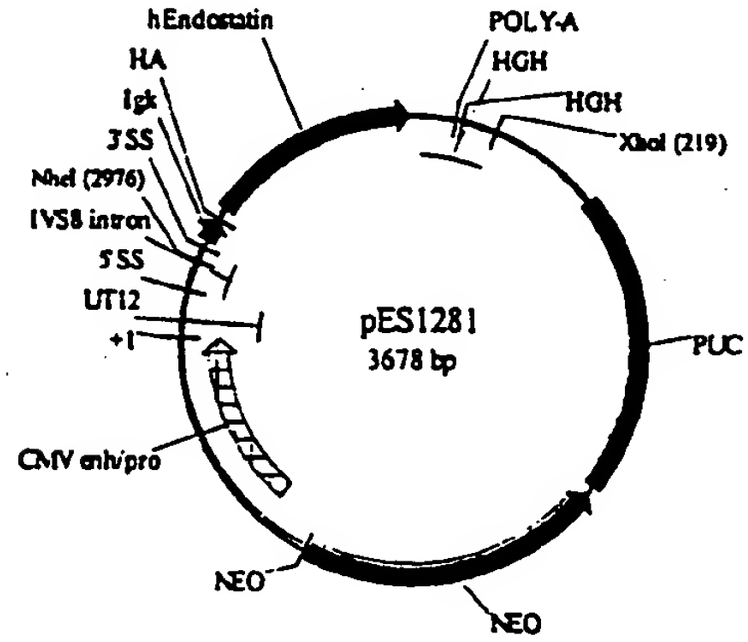
3804 bp
PUC


hEndostatin
HA
Igk
3'SS
NheI (2976)
IVS8 intron
5'SS
UT12
+1
CMV enh/pro
NEO
POLY-A
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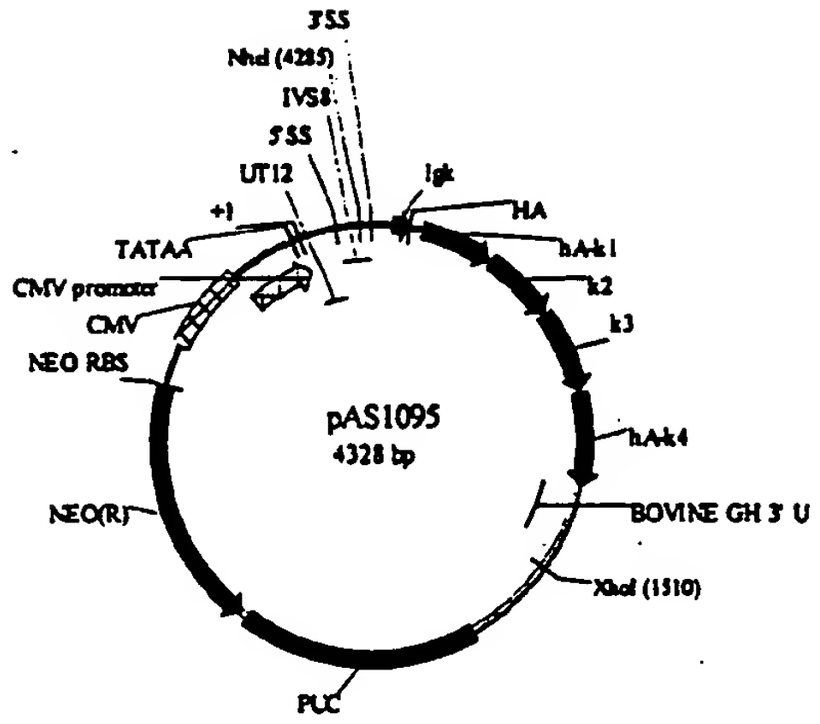
PUC
NEO

FIGURE 2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CMV
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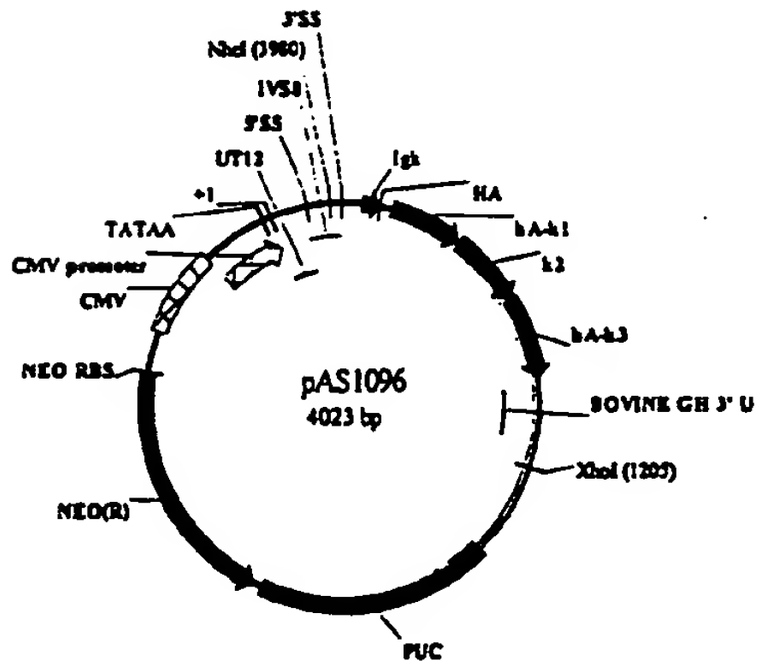
HA
hAk1
k2
k3
hAk4
BOVINE GH 3' U
XhoI (1510)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NheI (3980)
IVS8
5'SS
UT12
+1
TATAA
CMV promotor
CMV
NEO RBS
pAS1096
4023 bp
NEO(R)
PUC
Igk
HA
hAk1
k2
hAk3
BOVINE GH 3' U
XhoI (1205)

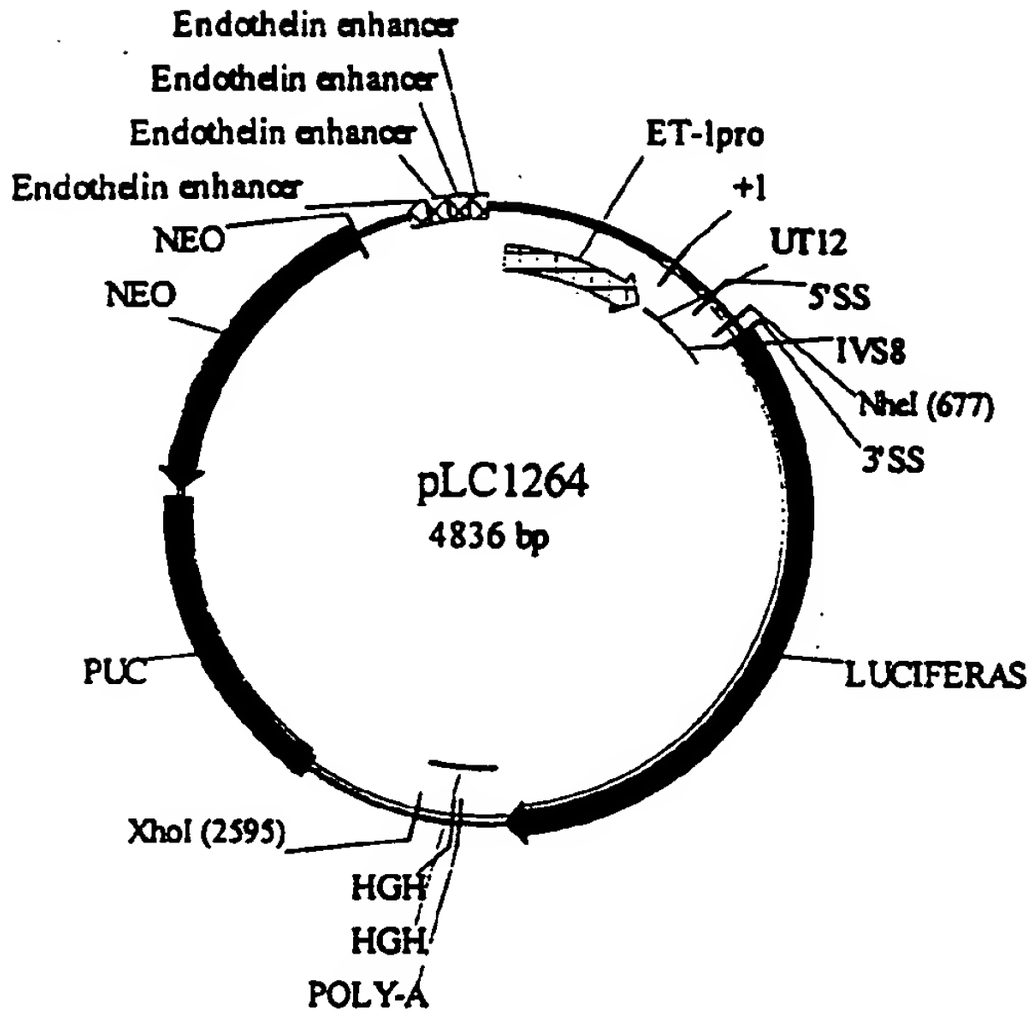
FIGURE 3

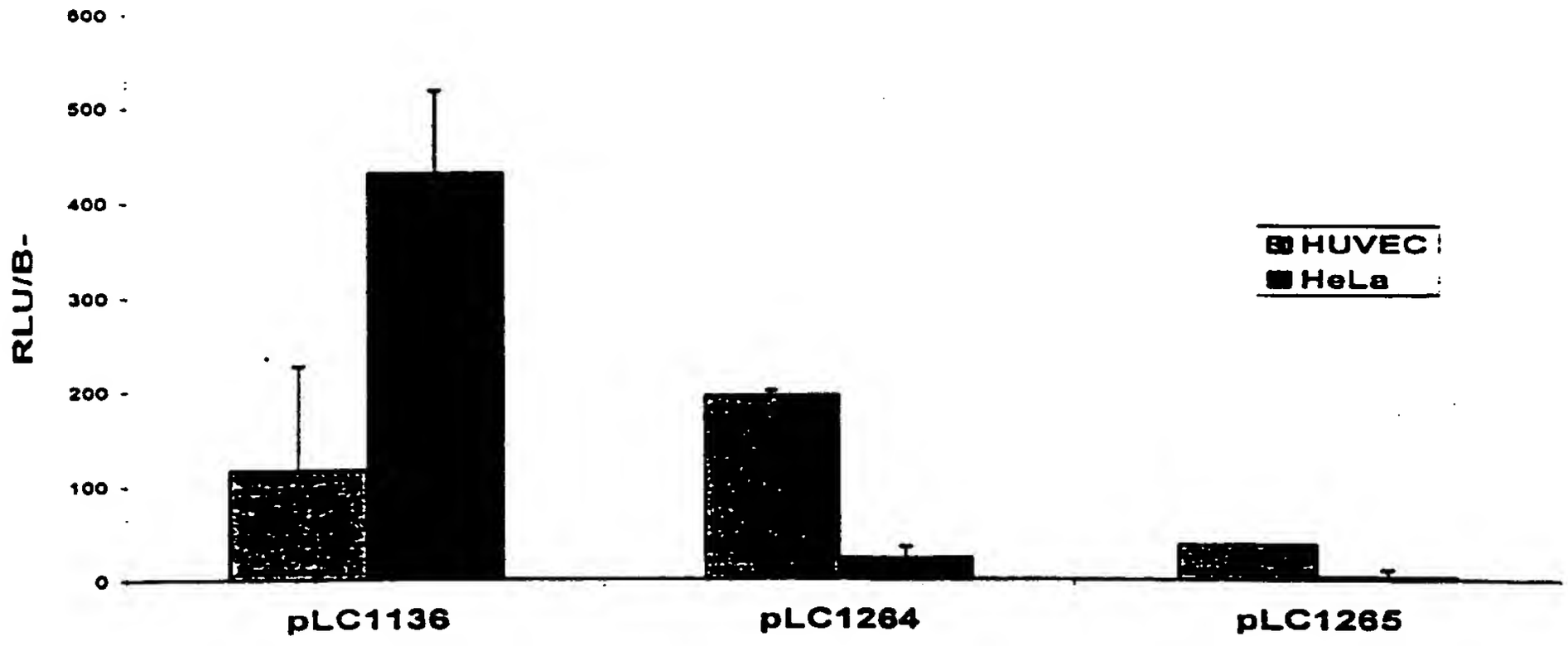
Promoter	Length (bp)	Position	Enhancer	5'UTR	3"UTR	Reporter	Plasmid name	Genbank (Accession number)	Ref.
ET-1	440	-250 to +170	CMV	UT12	HgH	luc	pLC1176	M25377	JBC 265:10446-50, 90
ET-1	440	-250 to +170	none	UT12	hGH	luc	pLC1177	M25377	JBC 265:10446-50, 90
ET-1	440	-250 to +170	4 copies of ET-1	UT12	hGH	luc	pLC1264	M25377	JBC 265:10446-50, 90
ET-1	440	-250 to +170	7 copies of ET-1	UT12	hGH	luc	pLC1265	M25377	JBC 265:10446-50, 90
flk-1	493	-225 to +268	CMV	UT12	hGH	luc	pLC1180	X89776	JBC 270:23111-8, 95
flk-1	493	-225 to +268	none	UT12	hGH	luc	pLC1181	X89776	JBC 270:23111-8, 95
Alpha -V	691	-522 to +169	CMV	UT12	hGH	luc	pLC1362	U07375	BBA 1219:228-32, 94
Alpha -V	691	-522 to +169	none	UT12	hGH	luc	pLC1363	U07375	BBA 1219:228-32, 94
Beta-3	586	-556 to +30	CMV	UT12	hGH	luc	pLC1364	L28832	Blood 83:668-76, 94
Beta-3	586	-556 to +30	none	UT12	hGH	luc	pLC1365	L28832	Blood 83:668-76, 94
ICAM-2	359	-336 to +23	CMV	UT12	hGH	luc	pLC1360		Patent WO 9711168 (Cowen)
ICAM-2	359	-336 to +23	none	UT12	hGH	luc	pLC1361		Patent WO 9711168 (Cowen)

Promoter	Length	Position	Enhancer	5'UTR	3'UTR	Plasmid	Reporter	Number
	327 bp	from -223 to +104	4 copies of ET-1	UT12	hGH	pLC1236	luciferase	X68303
cyc A	327 bp	from -223 to +105	7 copies of ET-1	UT12	hGH	pLC1237	luciferase	X68303
cyc A	327 bp	from -223 to +106	7 copies of ET-1	UT12	hGH	pCT1285	CAT	X68303
E2F1	323 bp	from -263 to +60	4 copies of ET-1	UT12	hGH	pLC1262	luciferase	S74230
E2F1	323 bp	from -263 to +60	7 copies of ET-1	UT12	hGH	pLC1263	luciferase	S74230
E2F1	323 bp	from -263 to +60	7 copies of ET-1	UT12	hGH	pCT1287	CAT	S74230
cdc6	137 bp	from -130 to +7	4 copies of ET-1	UT12	hGH	pLC1297	luciferase	none
cdc6	137 bp	from -130 to +7	7 copies of ET-1	UT12	hGH	pLC1298	luciferase	none
cdc6	137 bp	from -130 to +7	4 copies of ET-1	UT12	hGH	pCT1356	CAT	none
cdc6	137 bp	from -130 to +7	7 copies of ET-1	UT12	hGH	pCT1357	CAT	none

5 Endothelin enhancer (ET-1) 43 bp -364 to -320 **REF.**
JBC 272:32613-22, 51

FIGURE 4


Endothelin enhancer
Endothelin enhancer
Endothelin enhancer
Endothelin enhancer
ET-1pro
+1
UT12
5'SS
IVS8
NheI (677)
3'SS
NEO
NEO
pLC1264
4836 bp
PUC
LUCIFERAS
XhoI (2595)
HGH
HGH
POLY-A


Luciferase Activity in HUVECs and HeLas
RLU/B-
600
500
400
300
200
100
0
HUVEC
HeLa
pLC1136
pLC1264
pLC1265

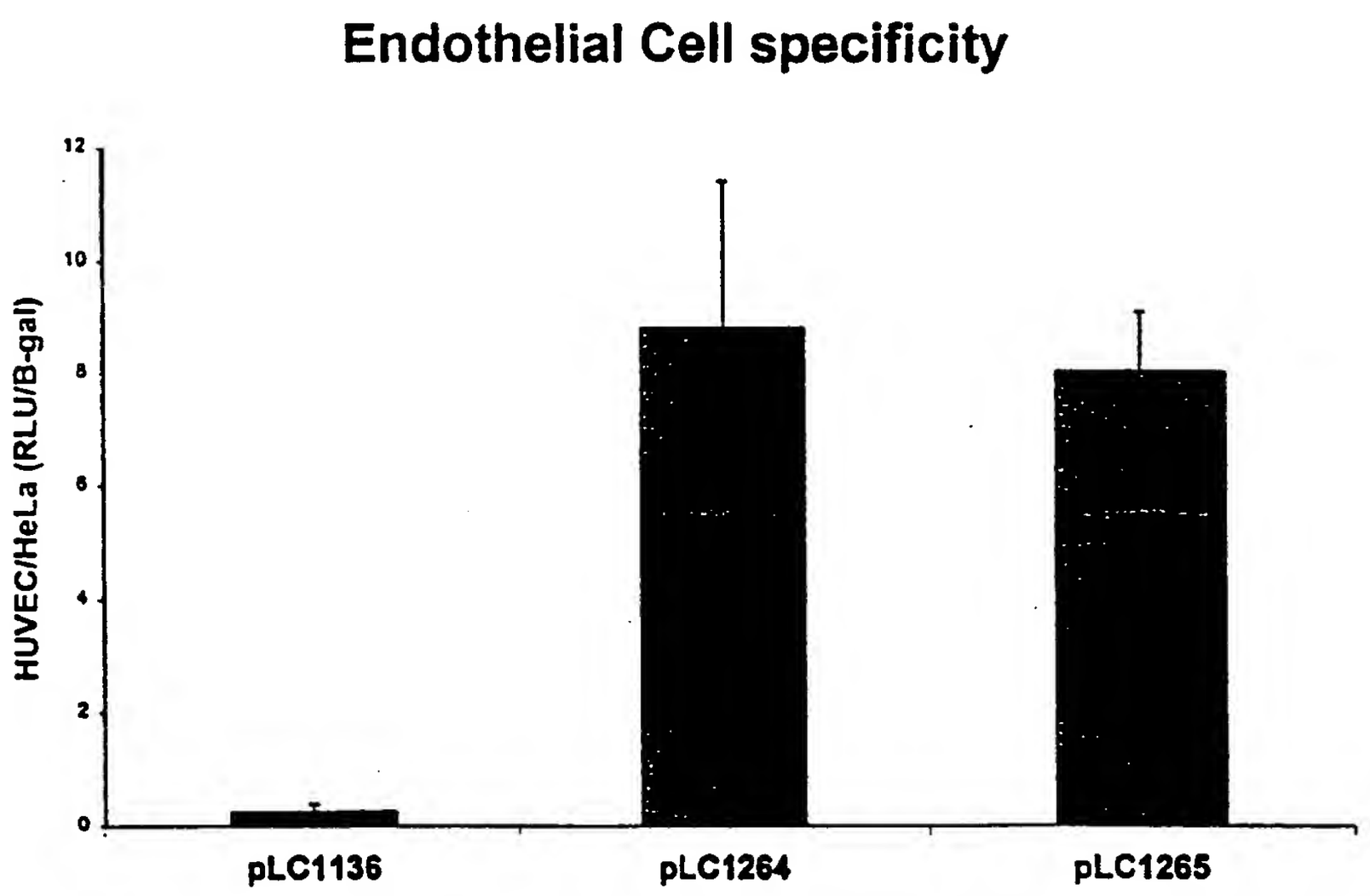

Endothelial Cell specificity
HUVEC/HeLa (RLU/B-gal)
12
10
8
6
4
2
0
pLC1136
pLC1264
pLC1265

FIGURE 5

FIGURE 6

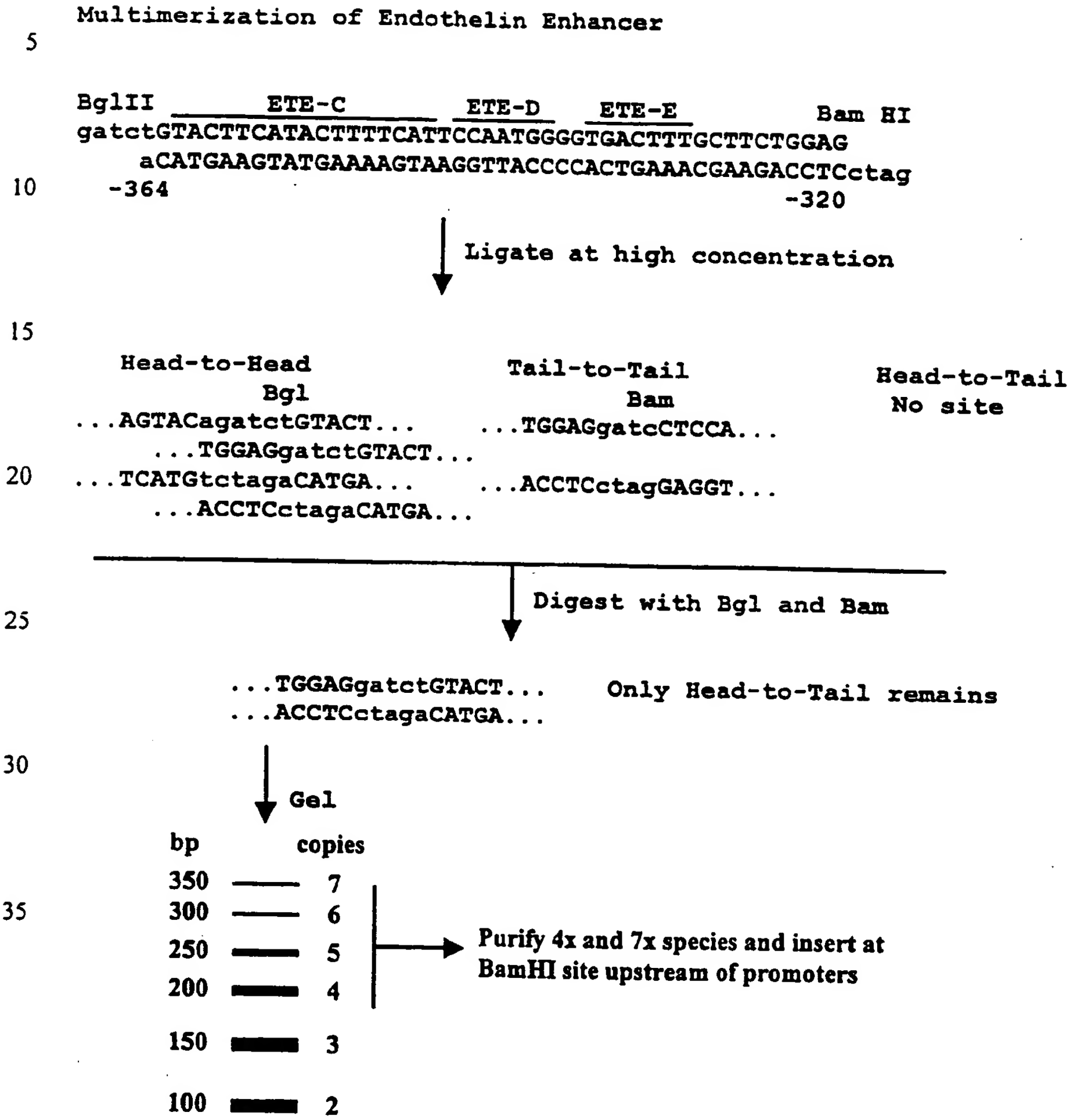
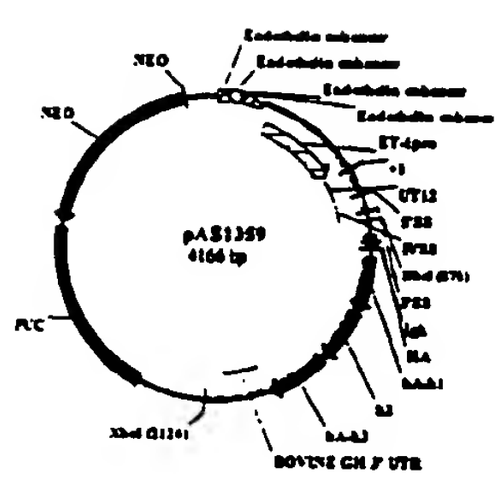
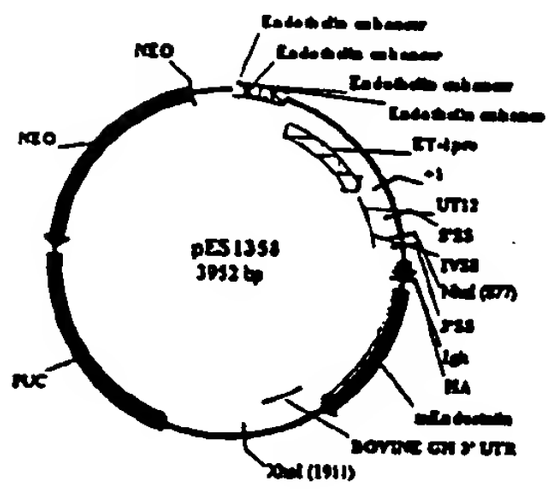

Multimerization of Endothelin Enhancer
BglII
ETE-C
ETE-D
ETE-E
Bam HI
gatctGTACTTCATACTTTTCATTCCAATGGGGTGACTTTGCTTCTGGAG
aCATGAAGTATGAAAAGTAAGGTTACCCCACTGAAACGAAGACCTCctag
-364
-320
Ligate at high concentration
Head-to-Head
Bgl
...AGTACagatctGTACT...
...TGGAGgatctGTACT...
...TCATGtctagaCATGA...
...ACCTCctagaCATGA...
Tail-to-Tail
Bam
...TGGAGgatcCTCCA...
...ACCTCctagGAGGT...
Head-to-Tail
No site
Digest with Bgl and Bam
...TGGAGgatctGTACT...
...ACCTCctagaCATGA...
Only Head-to-Tail remains
Gel
bp
copies
350
7
300
6
250
5
200
4
150
3
100
2
Purify 4x and 7x species and insert at
BamHI site upstream of promoters

FIGURE 7


NEO
pAS1359
4166 bp
PUC
BOVINE GH 3' UTR


NEO
NEO
pES1358
3952 bp
PUC
BOVINE GH 3' UTR
XbaI (1911)

In vitro expression of bioactive Endostatin

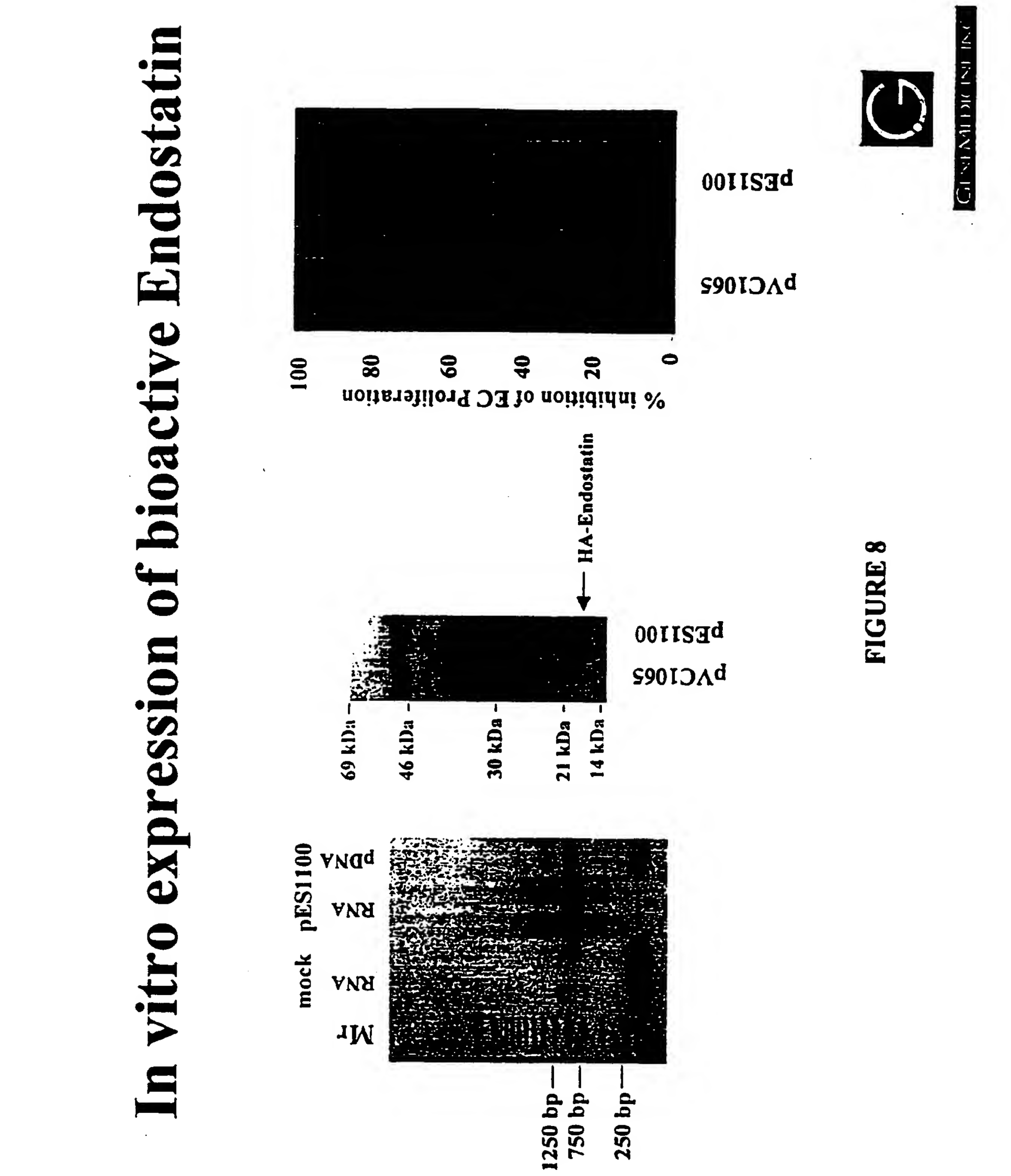


FIGURE 8

Endostatin/PVP inhibits Renca tumor

Treatment	Tumor on day10 (7 treatments) Rejection[a]	Volume (mm^3)[b]	Survival on day 24
Control	0/8	117	3/8 (37%)
Vector 96 ug	0/8	46	6/8 (75%)
Endostatin 96 ug	3/8	22	7/8 (87%)[c]
Endostatin 24 ug	4/8	23	8/8 (100%)[c]

a. No tumor or misc. tumor

b. Mean tumor volume of 8 mice

c. No tumor has come back by day 24

FIGURE 9

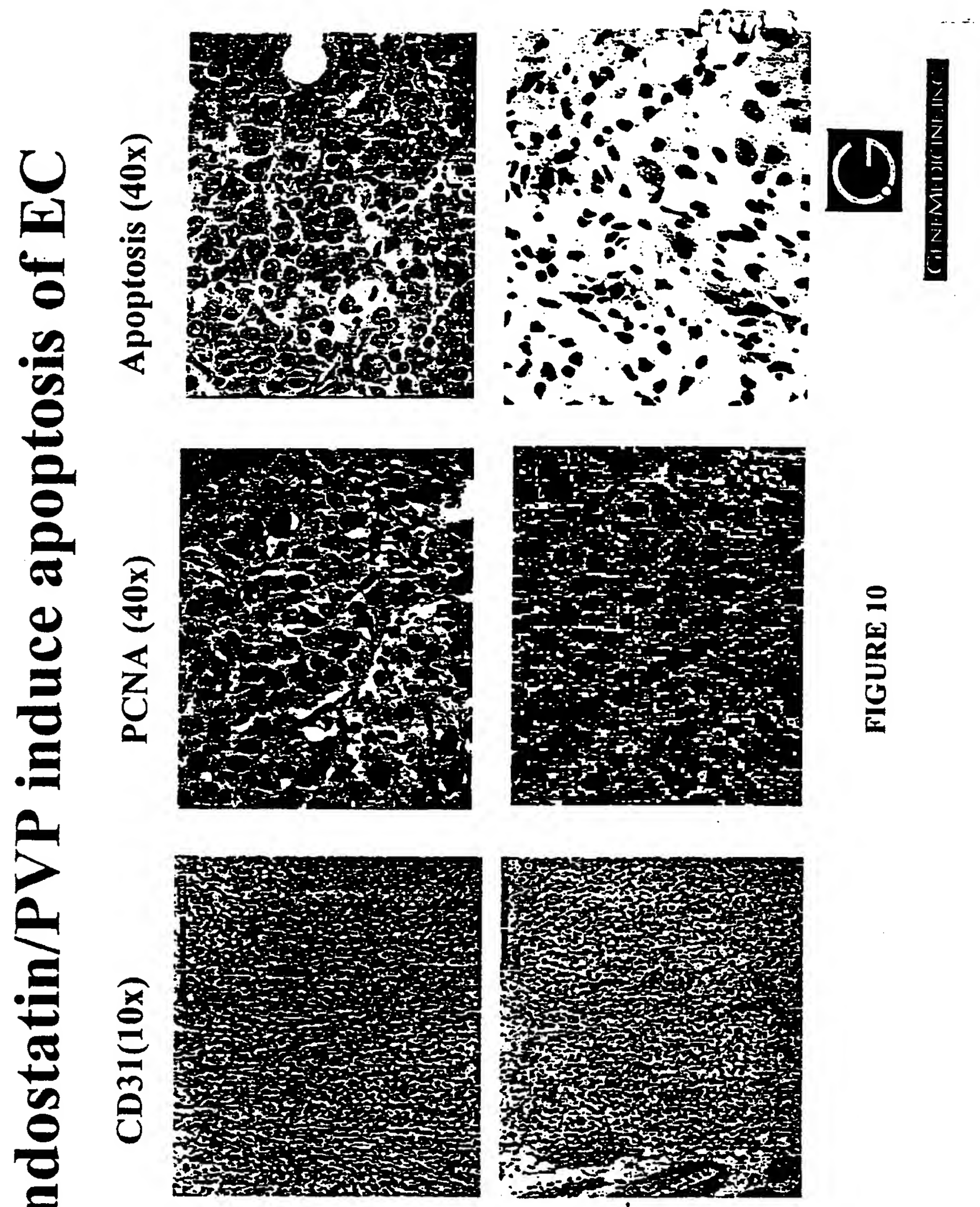
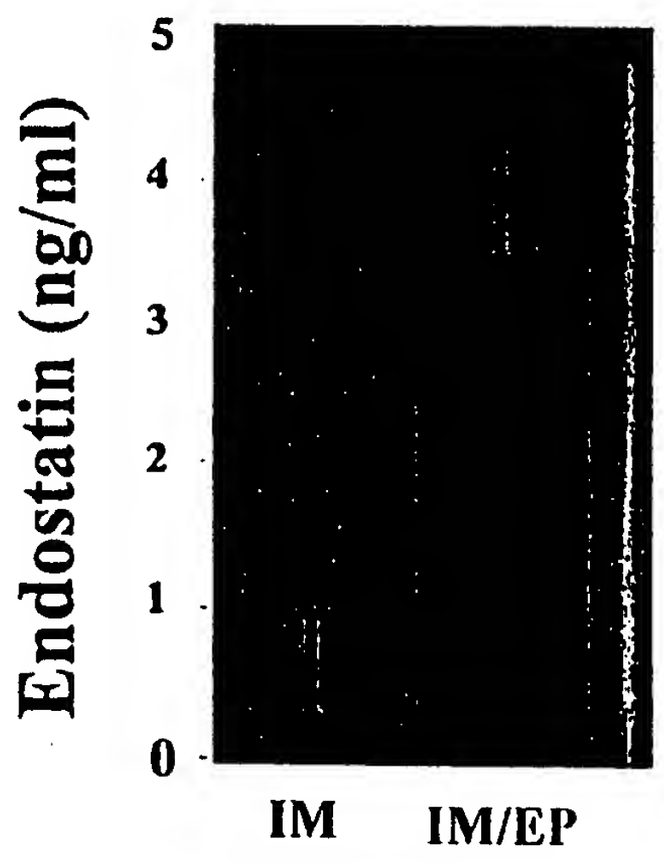
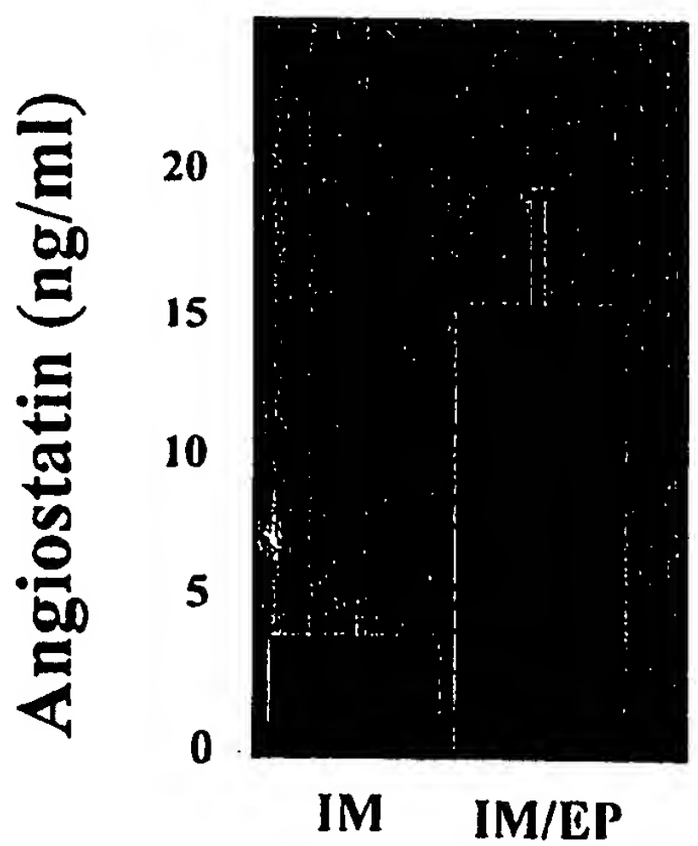

Endostatin/PVP induce apoptosis of EC
Apoptosis (40x)
PCNA (40x)
CD31(10x)
rowing Tumor
egressing Tumor
GENMEDICINE INC

FIGURE 10

Endostatin and Angiostatin expression in serum after intramuscular delivery



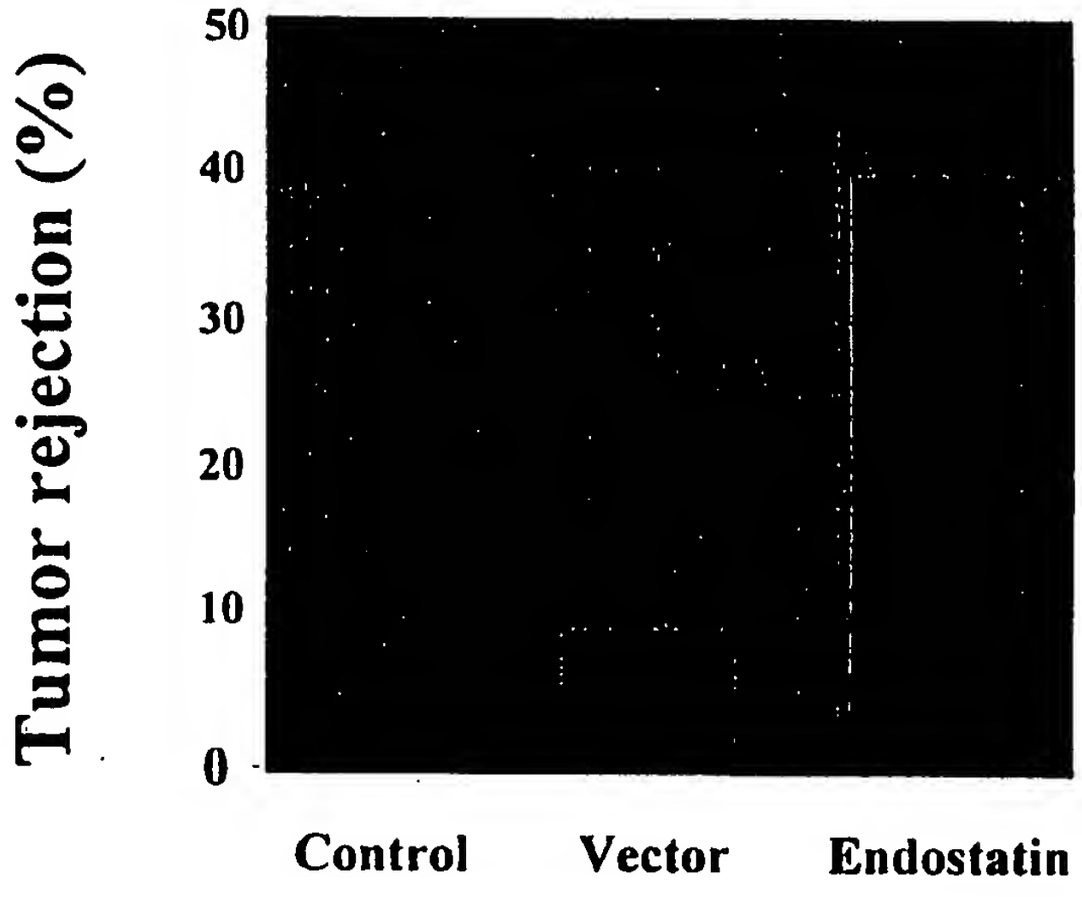


IM: DNA/PVP (240 μg), Tibialis and Gastrocnemius
Electroporation: 500 V, 2 min after injection
Harvest: serum on day 5

FIGURE 11

GENEMEDICINE, INC.

Endostatin/PVP inhibits sc Renca tumor after im delivery



IM/EP:	2x week , switch legs
Vector:	pVC0612/PVP (120 μg)
Endostatin	pES1100/PVP (120 μg)

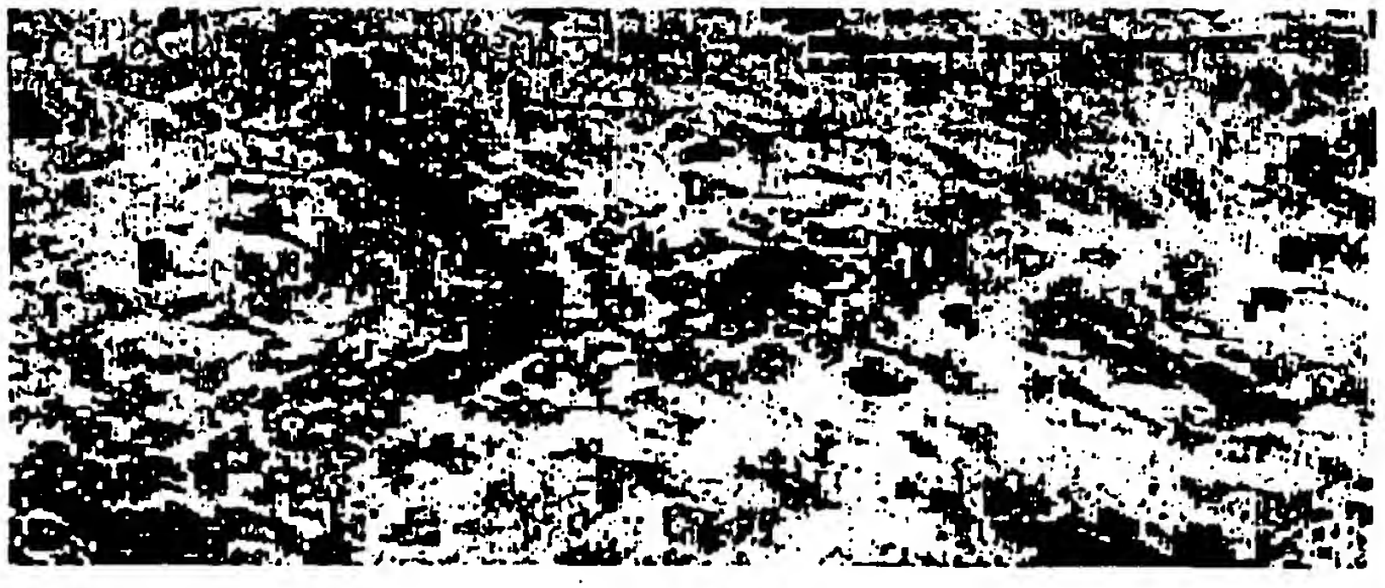
FIGURE 12

GENEMEDICINE,INC.

Endostatin/PVP inhibits sc Renca tumor after im delivery

Growing tumor

Regressing tumor



GENEMEDICINE INC

FIGURE 13

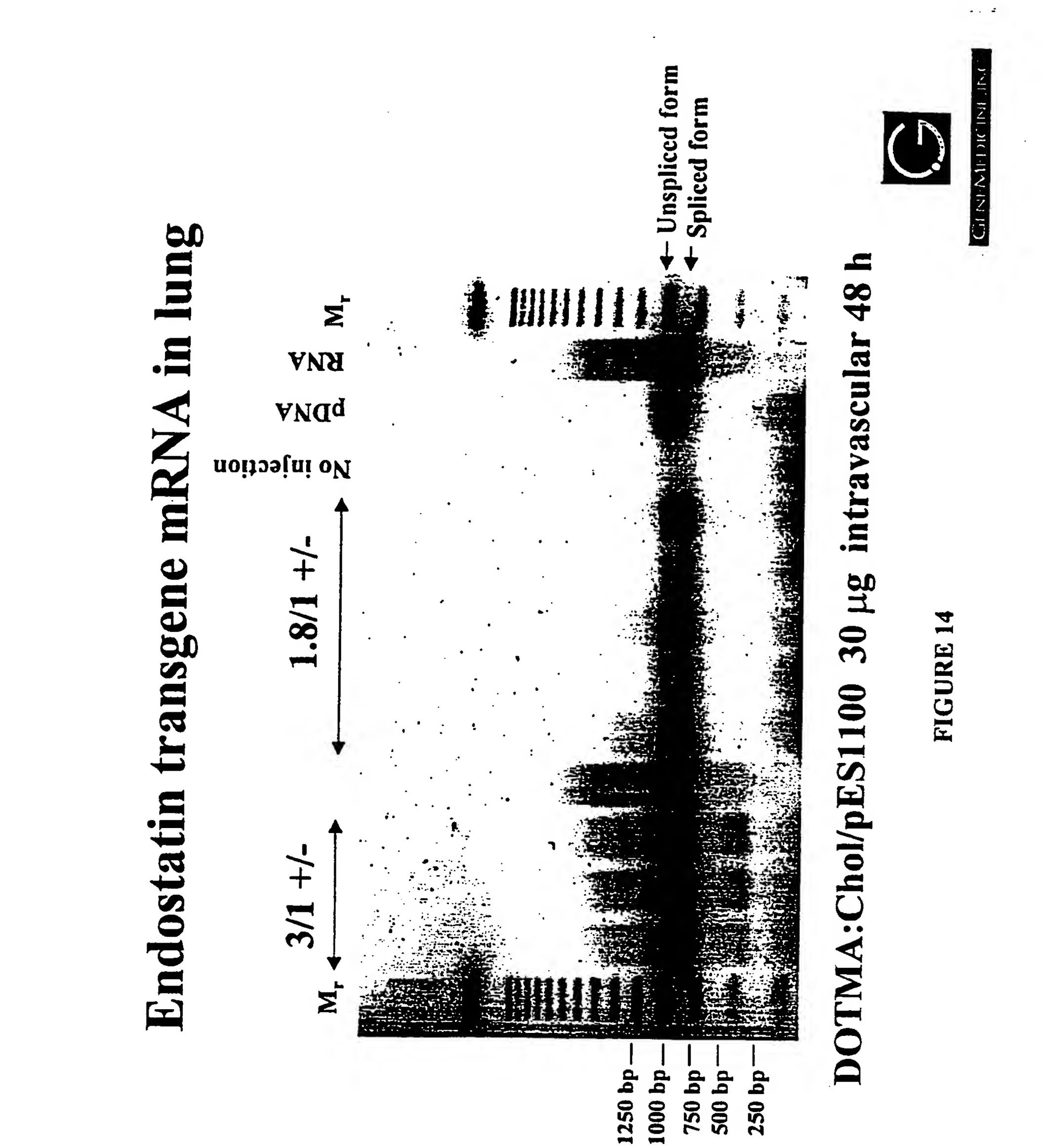

Endostatin transgene mRNA in lung
DOTMA:Chol/pES1100 30 μg intravascular 48 h
1.8/1 +/-
3/1 +/-
No injection
pDNA
RNA
M_r
M_r
1250 bp
1000 bp
750 bp
500 bp
250 bp
← Unspliced form
← Spliced form
GENEMEDICINE, INC.

FIGURE 14

Mouse cornea angiogenesis assay

Gene medicine **Control**

FIGURE 15

GENEMEDICINE, INC.

Codon Frequency human_high.doc

Codon usage for human (highly expressed) genes 1/24/91

AmAcid	Codon	Number	/1000	Fraction
Gly	GGG	905 00	18 76	0 24
Gly	GGA	523 00	10 85	0 14
Gly	GGT	441 00	9 14	0 12
Gly	GGC	1867 00	38 70	0 50
Glu	GAG	2420 00	50 16	0 75
Glu	GAA	792 00	16 42	0 25
Asp	GAT	592 00	12.27	0 25
Asp	GAC	1821 00	37 75	0 75
Val	GTG	1866 00	38 68	0 64
Val	GTA	134 00	2 78	0 05
Val	GTT	198 00	4 10	0 01
Val	GTC	728 00	15 09	0 25
Ala	GCG	632 00	13 31	0 17
Ala	GCA	488 00	10 12	0 13
Ala	GCT	654 00	13 56	0 17
Ala	GCC	2057 00	42 64	0 53
Arg	AGG	512 00	10 61	0 18
Arg	AGA	298 00	6 18	0 10
Ser	AGT	354 00	7 34	0 10
Ser	AGC	1171 00	24 27	0 34
Lys	AAG	2119 00	43 88	0 82
Lys	AAA	471 00	9 76	0 18
Asn	AAT	314 00	6 51	0 22
Asn	AAC	1120 00	23 22	0 78
Met	ATG	1077 00	22 32	1 00
Ile	ATA	88 00	1 82	0 05
Ile	ATT	335 00	6 53	0 18
Ile	ATC	1369 00	28 38	0 17
Thr	ACG	405 00	8 40	0 15
Thr	ACA	373 00	7 73	0 14
Thr	ACT	358 00	7 42	0 14
Thr	ACC	1502 00	31 13	0 57
Trp	TGG	652 00	13 51	1 00
End	TGA	109 00	2 26	0 55
Cys	TGT	325 00	6 74	0 32
Cys	TGC	706 00	14 63	0 68
End	TAG	42 00	0 87	0 21
End	TAA	46 00	0 95	0 23
Tyr	TAT	360 00	7 46	0 36
Tyr	TAC	1042 00	21 60	0 74
Leu	TTG	313 00	6 49	0 06
Leu	TTA	76 00	1 58	0 02
Phe	TTT	336 00	6 96	0 20
Phe	TTC	1377 00	28 54	0 80
Ser	TCG	325 00	6 74	0 06
Ser	TCA	165 00	3 42	0 05
Ser	TCT	450 00	9 33	0 13
Ser	TCC	958 00	19 86	0 28
Arg	CGG	611 00	12 67	0 21
Arg	CGA	183 00	3 79	0 06
Arg	CGT	210 00	4 35	0 07
Arg	CGC	1058 00	22 51	0 37
Gln	CAG	3020 00	61.87	0 83
Gln	CAA	583 00	5 87	0 12
His	CAT	234 00	4 85	0 21
His	CAC	870 00	18 03	0 79
Leu	CTG	2864 00	59 78	0 58
Leu	CTA	166 00	3 44	0 03
Leu	CTT	238 00	4 91	0 05
Leu	CTC	1276 00	26 45	0 26
Pro	CCG	432 00	8 99	0 17
Pro	CCA	454 00	9 45	0 18
Pro	CCT	568 00	11.77	0 19
Pro	CCC	1410 00	29 23	0 48

FIGURE 16

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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A3

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(30) Priority Data:
60/094,375 27 July 1998 (27.07.98) US

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(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MIN, Wang [CN/US]; 46 S. Acacia Park Circle, The Woodlands, TX 77385 (US). SZYMANSKI, Paul [US/US]; 10600 Six Pines Drive #515, The Woodlands, TX 77380 (US). MEHRENS, Dorothy [US/US]; 1711 Havelock Drive, Spring, TX 77386 (US). RALSTON, Robert [US/US]; 6 Lake Leaf Place, The Woodlands, TX 77381 (US). SULLIVAN, Sean [US/US]; 99 South Flagstone Path Circle, The Woodlands, TX 77381 (US).

(74) Agent: WARBURG, Richard, J.; Lyon & Lyon LLP, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071–2066 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

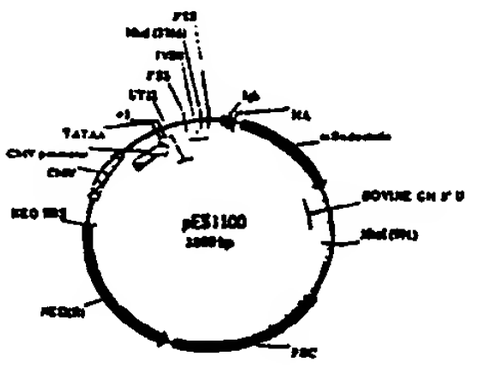
Published
With international search report.

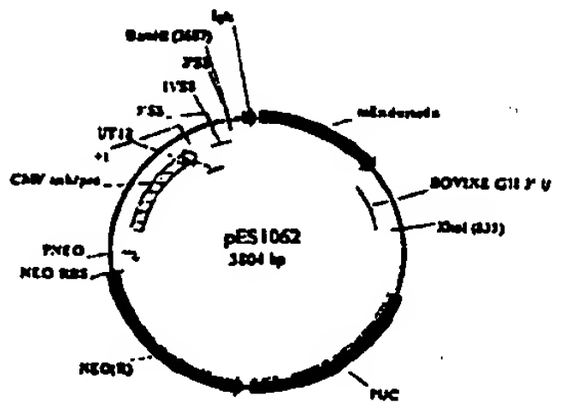
(88) Date of publication of the international search report:
22 June 2000 (22.06.00)

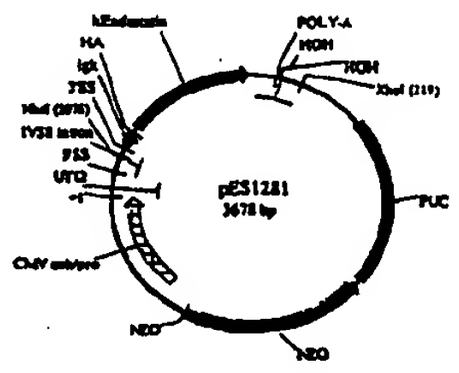
(54) Title: ANTI-ANGIOGENESIS PLASMIDS AND DELIVERY SYSTEMS, AND METHODS OF MAKING AND USING THE SAME

(57) Abstract

The present invention relates to gene delivery and gene therapy, and provides novel nucleic acid constructs for expression of anti-angiogenic agents in a mammal, formualtions for delivery that incorporate a nucleic acid construct for expression, and methods for preparing and using such constructs and formulations. In particular, this invention relates to plasmid constructs for delivery of therapeutic anti-angiogenic encoding nucleic acids to cells in order to modulate tumor activity, methods of using those constructs (including combination therapy with other agents, such as cytokines, preferably IL–12), as well as methods for preparing such constructs.







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INTERNATIONAL SEARCH REPORT

Inter ıal Application No
PCT/US 99/16388

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/85 C12N15/55 C12N15/12 C12N15/88 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BLEZINGER P ET AL: "Systemic inhibition of tumor growth and tumor metastases by intramuscular administration of the endostatin gene." NATURE BIOTECHNOLOGY, (1999 APR) 17 (4) 343-8., XP000857410 the whole document ---	1,3, 27-30, 34, 53-56, 61-77
P,X	BLEZINGER P ET AL: "Intratracheal administration of interleukin 12 plasmid-cationic lipid complexes inhibits murine lung metastases." HUMAN GENE THERAPY, (1999 MAR 20) 10 (5) 723-31., XP000862836 the whole document ---	52,60,82

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
20 December 1999	05 04 2000

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern: al Application No
PCT/US 99/16388

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 06940 A (BEHRINGWERKE AG ;SEDLACEK HANS HARALD (DE); BOSSLET KLAUS (DE); MU) 7 March 1996 (1996-03-07)	1-4,7, 53,54, 61, 63-65, 68, 71-73,76
Y	page 10 -page 13 page 4 -page 7	24, 27-30, 34-38, 47, 50-52, 55,57, 58,60, 77-79, 82,83

Y	MUMPER R J ET AL: "Protective interactive noncondensing (PINC) polymers for enhanced plasmid distribution and expression in rat skeletal muscle" JOURNAL OF CONTROLLED RELEASE,NL,ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, vol. 52, no. 1-2, page 191-203 XP004113667 ISSN: 0168-3659 the whole document	27-30, 34,50, 55,58, 77,78

X	EP 0 819 758 A (MIXSON ARCHIBALD JAMES) 21 January 1998 (1998-01-21)	52,60,82
Y	page 3 -page 5; examples 1-3	35,37, 47,50, 52,60, 79,82

X	THURSTON G ET AL: "Cationic liposomes target angiogenic endothelial cells in tumors and chronic inflammation in mice" THE JOURNAL OF CLINICAL INVESTIGATION, vol. 101, 1 April 1998 (1998-04-01), pages 1401-1403, XP000863045	52,60,82
Y	abstract	35,36, 38,50, 57,58

P,X	CHEN Q R ET AL: "Liposomes complexed to plasmids encoding angiostatin and endostatin inhibit breast cancer in nude mice." CANCER RESEARCH, (1999 JUL 15) 59 (14) 3308-12., XP000857407 the whole document	52,57, 60,82,83

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INTERNATIONAL SEARCH REPORT

Inter ial Application No
PCT/US 99/16388

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EP 0 921 193 A (MIXSON ARCHIBALD JAMES) 9 June 1999 (1999-06-09) page 8 -page 10; examples 1-13	35,37, 47,52, 60,79,82
Y	WO 97 15666 A (CHILDRENS MEDICAL CENTER) 1 May 1997 (1997-05-01) page 11; figure 14 page 45 -page 46	24,51,83
A	BOEHM T ET AL: "Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance [see comments]." NATURE, (1997 NOV 27) 390 (6658) 404-7., XP000857413	
A	O'REILLY M S ET AL: "ENDOSTATIN: AN ENDOGENOUS INHIBITOR OF ANGIOGENESIS AND TUMOR GROWTH" CELL,US,CELL PRESS, CAMBRIDGE, NA, vol. 88, no. 2, page 277-285 XP000652213 ISSN: 0092-8674	
A	O'REILLY M ET AL: "ANGIOSTATIN A NOVEL ANGIOGENESIS INHIBITOR THAT MEDIATES THE SUPPRESSION OF METASTASES BY A LEWIS LUNG CARCINOMA" CELL,US,CELL PRESS, CAMBRIDGE, NA, vol. 79, page 315-328 XP002015254 ISSN: 0092-8674	

INTERNATIONAL SEARCH REPORT

In.. ational application No.
PCT/US 99/ 16388

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 61-67,71-75,77-83 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet, subject 1.

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-4,7-33,35-49,53,54,57,61-77,79

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99/16388

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: in part: 1-4,7-33,35-49,53,54,57,61-77,79

Plasmid comprising an endothelin-1 (ET-1) promoter transcriptionally linked to an antiangiogenic coding sequence; cell transfected with said plasmid; composition comprising said plasmid and a protective, interactive non-condensing compound; composition comprising said plasmid and a cationic lipid; method for making said plasmid; method for treatment of a mammalian condition or disease comprising administring to a mammal said plasmid; method for transfection of a cell in situ with said plasmid; method for delivery and expression of an antiangiogenic gene in a plurality of cells by using said plasmid; method for treating a disease or condition comprising the steps of transfecting a cell in situ with said plasmid;

1.1. Claims: 34,51,55,56,59,78,81; in part 50,58,80
Composition comprising a protective, interactive non-condensing compound and a plasmid comprising an anti-angiogenic coding sequence; method for making said composition; method for treatment of a mammalinan condition or disease comprising administering to a mammal said composition

1.2. Claims: 52,60,82; ; in part 50,58,80
Composition comprising a plasmid comprising an anti-angiogenic coding sequence and a cationic lipid with a neutral co-lipid; method for making said composition; method for treatment of a mammalian condition or disease, comprising administering to a mammal said composition

1.3. Claim : 83
Method for treatment of a mammalian condition or disease , comprising administering to a mammal a composition of a first plasmid comprising an angiostatin coding sequence and a second plasmid comprising an endostatin coding sequence

2. Claims: in part: 1-4,7-33,35-49,53,54,57,61-77,79

Plasmid comprising a flk-1 promoter transcriptionally linked to an antiangiogenic coding sequence; cell transfected with said plasmid; composition comprising said plasmid and a protective, interactive non-condensing compound; composition comprising said plasmid and a cationic lipid; method for making said plasmid; method for treatment of a mammalian condition or disease comprising administring to a mammal said plasmid; method for transfection of a cell in situ with said plasmid; method for delivery and expression of an antiangiogenic gene in a plurality of cells by using said

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

plasmid; method for treating a disease or condition comprising the steps of transfecting a cell in situ with said plasmid;

3. Claims: in part: 1-4,7-33,35-49,53,54,57,61-77,79

Plasmid comprising an Alpha-V promoter transcriptionally linked to an antiangiogenic coding sequence; cell transfected with said plasmid; composition comprising said plasmid and a protective, interactive non-condensing compound; composition comprising said plasmid and a cationic lipid; method for making said plasmid; method for treatment of a mammalian condition or disease comprising administring to a mammal said plasmid; method for transfection of a cell in situ with said plasmid; method for delivery and expression of an antiangiogenic gene in a plurality of cells by using said plasmid; method for treating a disease or condition comprising the steps of transfecting a cell in situ with said plasmid;

4. Claims: in part: 1-4,7-33,35-49,53,54,57,61-77,79

Plasmid comprising a Beta-3 promoter transcriptionally linked to an antiangiogenic coding sequence; cell transfected with said plasmid; composition comprising said plasmid and a protective, interactive non-condensing compound; composition comprising said plasmid and a cationic lipid; method for making said plasmid; method for treatment of a mammalian condition or disease comprising administring to a mammal said plasmid; method for transfection of a cell in situ with said plasmid; method for delivery and expression of an antiangiogenic gene in a plurality of cells by using said plasmid; method for treating a disease or condition comprising the steps of transfecting a cell in situ with said plasmid;

5. Claims: in part: 1-4,7-33,35-49,53,54,57,61-77,79

Plasmid comprising a ICAM-2 promoter transcriptionally linked to an antiangiogenic coding sequence; cell transfected with said plasmid; composition comprising said plasmid and a protective, interactive non-condensing compound; composition comprising said plasmid and a cationic lipid; method for making said plasmid; method for treatment of a mammalian condition or disease comprising administring to a mammal said plasmid; method for transfection of a cell in situ with said plasmid; method for delivery and expression of an antiangiogenic gene in a plurality of cells by using said plasmid; method for treating a disease or condition comprising the steps of transfecting a cell in situ with said plasmid;

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

6. Claims: in part: 1-4,7-33,35-49,53,54,57,61-77,79

Plasmid comprising a cyc A promoter transcriptionally linked to an antiangiogenic coding sequence; cell transfected with said plasmid; composition comprising said plasmid and a protective, interactive non-condensing compound; composition comprising said plasmid and a cationic lipid; method for making said plasmid; method for treatment of a mammalian condition or disease comprising administring to a mammal said plasmid; method for transfection of a cell in situ with said plasmid; method for delivery and expression of an antiangiogenic gene in a plurality of cells by using said plasmid; method for treating a disease or condition comprising the steps of transfecting a cell in situ with said plasmid;

7. Claims: in part: 1-4,7-33,35-49,53,54,57,61-77,79

Plasmid comprising an E2F1 promoter transcriptionally linked to an antiangiogenic coding sequence; cell transfected with said plasmid; composition comprising said plasmid and a protective, interactive non-condensing compound; composition comprising said plasmid and a cationic lipid; method for making said plasmid; method for treatment of a mammalian condition or disease comprising administring to a mammal said plasmid; method for transfection of a cell in situ with said plasmid; method for delivery and expression of an antiangiogenic gene in a plurality of cells by using said plasmid; method for treating a disease or condition comprising the steps of transfecting a cell in situ with said plasmid;

8. Claims: in part: 1-4,7-33,35-49,53,54,57,61-77,79

Plasmid comprising an cdc6 promoter transcriptionally linked to an antiangiogenic coding sequence; cell transfected with said plasmid; composition comprising said plasmid and a protective, interactive non-condensing compound; composition comprising said plasmid and a cationic lipid; method for making said plasmid; method for treatment of a mammalian condition or disease comprising administring to a mammal said plasmid; method for transfection of a cell in situ with said plasmid; method for delivery and expression of an antiangiogenic gene in a plurality of cells by using said plasmid; method for treating a disease or condition comprising the steps of transfecting a cell in situ with said plasmid;

9. Claims: in part: 1,5-33,35-49,53,54,57,61-77,79

Plasmid comprising a CMV enhancer transcriptionally linked

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

to an antiangiogenic coding sequence; cell transfected with said plasmid; composition comprising said plasmid and a protective, interactive non-condensing compound; composition comprising said plasmid and a cationic lipid; method for making said plasmid; method for treatment of a mammalian condition or disease comprising administring to a mammal said plasmid; method for transfection of a cell in situ with said plasmid; method for delivery and expression of an antiangiogenic gene in a plurality of cells by using said plasmid; method for treating a disease or condition comprising the steps of transfecting a cell in situ with said plasmid;

10. Claims: in part: 1,5-33,35-49,53,54,57,61-77,79

Plasmid comprising four copies of ET-1 enhancer transcriptionally linked to an antiangiogenic coding sequence; cell transfected with said plasmid; composition comprising said plasmid and a protective, interactive non-condensing compound; composition comprising said plasmid and a cationic lipid; method for making said plasmid; method for treatment of a mammalian condition or disease comprising administring to a mammal said plasmid; method for transfection of a cell in situ with said plasmid; method for delivery and expression of an antiangiogenic gene in a plurality of cells by using said plasmid; method for treating a disease or condition comprising the steps of transfecting a cell in situ with said plasmid;

11. Claims: in part: 1,5-33,35-49,53,54,57,61-77,79

Plasmid comprising seven copies of ET-1 enhancer transcriptionally linked to an antiangiogenic coding sequence; cell transfected with said plasmid; composition comprising said plasmid and a protective, interactive non-condensing compound; composition comprising said plasmid and a cationic lipid; method for making said plasmid; method for treatment of a mammalian condition or disease comprising administring to a mammal said plasmid; method for transfection of a cell in situ with said plasmid; method for delivery and expression of an antiangiogenic gene in a plurality of cells by using said plasmid; method for treating a disease or condition comprising the steps of transfecting a cell in situ with said plasmid;

INTERNATIONAL SEARCH REPORT

...rmation on patent family members

Interna... al Application No
PCT/US 99/16388

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